University of Alberta

The Biological and Immunological Aspect of the Host-Parasite Relationship of Goldfish,
Carassius auratus, Infected with *Trypanosoma danilewskyi*

by

Diane Rose Bienek ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta

Spring 2001

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-60274-5

Canada

University of Alberta

Library Release Form

Name of Author: Diane Rose Bienek

Title of Thesis: The Biological and Immunological Aspect of the Host-Parasite Relationship of Goldfish, *Carassius auratus*, Infected with *Trypanosoma danilewskyi*

Degree: Doctor of Philosophy

Year this Degree Granted: 2001

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Diane Bienek

Box 476
Smoky Lake, Alberta
T0A 3C0

Jan. 31, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'The Biological and Immunological Aspect of the Host-Parasite Relationship of Goldfish, *Carassius auratus,* Infected with *Trypanosoma danilewskyi.*' submitted by Diane Rose Bienek in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

Dr. M. Belosevic (Supervisor)

Dr. W.M. Samuel (Co-supervisor)

Dr. G.D. Armstrong

January 26/2005

Dr. J.P. Chang

Andrew Keddie
Dr. B.A. Keddie

Dr. M.E. Olson (External Examiner)

ABSTRACT

Trypanosoma danilewskyi is a parasitic protozoan that lives in tissues of fish from Europe and Asia. In aquaculture industry, the prevalence of *T. danilewskyi* infection has approached 100% in some populations. Because little is known about the biological association between the host and the parasite, it is difficult to establish appropriate control measures.

The objective of this research was to: 1) develop reliable *in vitro* procedures for cultivation of trypanosomes; and 2) examine components of the host-parasite relationship that influence the susceptibility of goldfish, *Carassius auratus*.

Serum-related factors in fish blood were required for long-term cultivation of *T. danilewskyi*, because medium containing mammalian serum (10% bovine or horse) or no serum failed to support growth. Bloodstream-forms were the predominant stage in cultures containing goldfish serum. Medium supplemented with soluble cell products, collected from macrophages isolated from infected or uninfected fish, supported parasite growth better ($P < 0.05$) than control medium. Similar experiments with macrophage (mammalian) or fibroblast cells (fish or mammalian) revealed that growth-enhancing activity was not specific to species or cell type.

As previously reported, fish acquired resistance after alleviation of the acute primary infection. Further, passive transfer of immune serum to naïve fish conferred protection against trypanosomes. These corroborative results led to an *in vitro* experiment that determined that blood leukocytes, derived from convalescent fish, were responsive to stimulation with trypanosome extract. Subsequently, I studied active immunization of goldfish with trypanosome extracts (whole dead, lysate, water-solubilized molecules, detergent-solubilized molecules, and excretory-secretory products) via different vehicles and routes. The main conclusions drawn from these experiments were: 1) intraperitoneal administration of excretory-secretory products in

Freund's complete adjuvant conferred protection ($P < 0.05$); 2) administration of other extracts incurred insignificant levels of protection or enhanced the susceptibility to the parasite; 3) the role of parasite-specific antibodies in eliminating trypanosomes seems unlikely, as there was no relationship between protection and anti-parasite antibody; 4) administration route (intravenous, intramuscular, or intraperitoneal) of immunization did not influence the outcome of the infection; and 5) of the five parameters examined, the prevalence of infection and parasite abundance were the best indicators of protection.

ACKNOWLEDGMENTS

No usual thanks are sufficient to acknowledge my debt to my supervisors, Drs. M. Belosevic and W. Samuel. I thank them for being an endless source of kindness and intellectual support. Similarly, I extend thanks to my committee members, Drs. G. Armstrong and J. Chang for their insights and contributions. I am grateful for their gift of time and attention.

NSERC and AHFMR operating grants to Dr. Belosevic provided funding for this research. Also, the Department of Biological Sciences, University of Alberta, provided financial support through teaching assistantships.

Dr. A. Shostak deserves thanks and acknowledgement for providing statistical consulting that was vital to data analyses. I appreciate his patience and willingness to answer questions. Also, thank you for reviewing parts of this thesis.

Special thanks to Mr. Bruce Wakeford for the care given to the fish used in this study. I am thankful to Dr. N. Stacey for providing tin foil barbs and common carp. Likewise, thanks to Drs. C. Paskowski and W. Tonn for providing space in the aquatic facility.

I am indebted to the other parasitology/immunology graduate students, Rebecca Guy, Norm Neumann, Matthew Lowry, Dan Barreda, and James Stafford, for serving as informal mentors. They kindly gave of their time and knowledge to assist me in my research. I am thankful to Debbie Plouffe for providing excellent technical assistance during the summer months of 1999. As well, Cezary Kucharski always offered genuine kindness and numerous helpful contributions, including but not limited to photography expertise.

Lastly, my gratitude to my parents, Rosemarie and Dr. Gerhard Bienek, is far ranging. They have been a continuous source of love, encouragement, and support. I treasure their advice and friendship. I'm everything I am, because they loved me.

TABLE OF CONTENTS

Chapter 1. Introduction and literature review 1

  Introduction 1

    Objectives 3

  Literature review 4

    History 4

    Life cycle 5

    Pathogenesis 6

    Control measures/therapeutics 7

    Immunobiology of parasitic fish protozoa 9

      Humoral immunity 10

      Complement 13

      Phagocytes 16

      T-cell-mediated responses 17

      Nonspecific cytotoxic cells 18

    Antigens of *Trypanosoma* spp. 19

      Major surface antigens 19

      Parasite-released products 20

    Metabolism and growth of trypanosomes 21

      Nutrient acquisition 21

      Energy metabolism 22

    Substances potentiating growth of *Trypanosoma* spp. 23

      Influence of cells on the cultivation and differentiation of trypanosomes..24

Influence of molecules on the cultivation and differentiation of trypanosomes..25

- Monosaccharides..25
- Amino acids..27
- Purine bases, nucleotides, and nucleosides..28
- Iron complexes..28
- Cytokines..29

Chapter 2. General materials and methods..33

- Fish..33
- Parasite..33
- Maintaining parasite stock cultures..33
- Infection of fish..34
- Assessment of infection..34
- Purification of parasites from blood..35
- Preparation of fish serum/plasma..35
- Isolation of peripheral blood leukocytes..36
- Kidney leukocytes..36
  - Isolation of leukocytes..36
  - Production of macrophage-conditioned medium..37
- Cell lines..37
  - Maintenance..37
  - Production of cell-conditioned medium..37

Cultivation in cell-conditioned medium 37
Enumeration of parasites 38
  Light Microscopy 38
  Flow cytometry 38
XTT viability assay 38
Wright's stain technique 38
Antigen preparation 39
  Sodium perchlorate treatment 39
  Preparation of trypanosome lysate 39
  Preparation of water-soluble extracts 39
  Preparation of detergent-soluble extracts 39
  Collection of excretory-secretory products 40
Fish immunization procedure 40
Determination of packed red cell volume 41
Production of anti-carp immunoglobulin 41
Immunological assays 42
  Enzyme-linked immunosorbent assay 42
  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis 43
  Immunoblot analyses 43
Passive transfer of immunity 43
Estimation of protein concentration 44
Statistical analyses 44
Chapter 3. Characterization of the course of infection 50

Introduction 50

Experimental design 50

Results 51

Discussion 52

Chapter 4. Cultivation of *Trypanosoma danilewskyi in vitro* *58*

Introduction 58

Experimental design 59

Comparative assessment of sera 59

Morphometric study of cultured trypanosomes 61

Comparative assessment of cell-conditioned medium 61

Results 62

Comparative assessment of sera 62

Morphometric study of cultured trypanosomes 64

Comparative assessment of cell-conditioned medium 65

Fish kidney leukocytes 65

Mammalian macrophages 67

Fibroblasts 68

Discussion 69

Chapter 5. Analysis of active immunization of goldfish for protection against *Trypanosoma danilewskyi* 93

Introduction 93

Experimental design 94

Active immunization 94

Antigen-induced proliferation of leukocytes 95

Passive transfer of immunity 95

Results 95

Active immunization 95

Excretory/secretory products 96

Intravenous administration 96

Intramuscular administration 97

Intraperitoneal administration (no adjuvant) 98

Intraperitoneal administration (Freund's incomplete adjuvant) 100

Intraperitoneal administration (Freund's complete adjuvant) 100

Gel electrophoresis/immunoblotting 102

Sodium perchlorate treatment 102

Intravenous administration 102

Intramuscular administration 104

Intraperitoneal administration (no adjuvant) 104

Intraperitoneal administration (Freund's adjuvant) 105

Infection dynamics of non-immunized control fish 107

Relative antibody response of non-immunized control fish 108

ES products 108

Sodium perchlorate-killed trypanosomes 109

Trypanosome lysate 109

Water-soluble extracts 109

Antigen-induced proliferation of leukocytes 110
Passive transfer of serum 110
Discussion 111
Immunization conferred protection against *T. danilewskyi* 112
Insignificant levels of protection 113
Immunization enhanced susceptibility to *T. danilewskyi* 118
Red cell volume/anemia 119
Administration route 121
Chapter 6. General discussion 148
Literature cited 157

LIST OF TABLES

Table 1-1. A general summary of classification, transmission, host range, and tissue affinity of fish protozoa 31

Table 2-1. Composition of goldfish leukocyte medium 46

Table 2-2. Optimal concentration of antigen and primary antibody used for the ELISA 47

Table 2-3. Statistical analyses performed on experimental data 48

Table 3-1. Parasite intensity in naïve goldfish that were inoculated with *Trypanosoma danilewskyi*. 55

Table 4-1. Effect of serum-free medium and horse serum on the *in vitro* growth of *Trypanosoma danilewskyi* 74

Table 4-2. *In vitro* growth of *Trypanosoma danilewskyi* in Transwell cell culture inserts containing kidney leukocytes derived from goldfish. 75

Table 4-3. Protein concentration of undiluted supernatants collected from mammalian macrophage (P388D.1) or fibroblast (L929) cell-lines 76

Table 4-4. *In vitro* growth of *Trypanosoma danilewskyi* in control medium or medium conditioned by mouse fibroblasts, L929 77

Table 4-5. *In vitro* growth of *Trypanosoma danilewskyi* in control medium or medium conditioned by fish fibroblasts, CCL71 78

Table 5-1. Percent of control value of the antibody response of fish immunized with excretory-secretory products of *Trypanosoma danilewskyi* 122

Table 5-2. Correlation coefficient (r) between parasitemia and the antibody response of fish immunized with excretory-secretory products of *Trypanosoma danilewskyi*. 123

Table 5-3. Survival of fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi*. 124

Table 5-4. Prevalence of infection in fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi* 125

Table 5-5. Abundance of parasites in fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi*. 126

Table 5-6. Correlation coefficient (r) between parasitemia and the antibody response of fish immunized with whole dead (sodium perchlorate-treated) *Trypanosoma danilewskyi*........ 127

Table 5-7. Relative antibody response of fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi*. 128

Table 5-8. Percent of control value of the antibody response of fish immunized with whole dead (sodium perchlorate-treated) *Trypanosoma danilewskyi* ... 129

Table 5-9. Red cell volume in blood of fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi*. 130

Table 5-10. Summary of results (parasitemia) obtained from experiments that evaluated the efficacy of parasite extracts and inoculum route on the susceptibility of goldfish to *Trypanosoma danilewskyi*. 131

Table 5-11. Summary of results (parasitemia) comparing the susceptibility of goldfish, administered control solution prior to *Trypanosoma danilewskyi* infection, with that of non-immunized infected goldfish.. 132

Table 5-12. Summary of results (ELISA values) obtained from experiments that evaluated the efficacy of immunization on the humoral immune response of goldfish, prior to infection with *Trypanosoma danilewskyi* 133

Table 5-13. Summary of results (ELISA values) obtained from experiments that evaluated the efficacy of parasite extracts and inoculum route on the susceptibility of goldfish to *Trypanosoma danilewskyi*. 134

Table 5-14. Summary of results (red cell volume) obtained from experiments that evaluated the efficacy of parasite extracts and inoculum route on the susceptibility of goldfish to *Trypanosoma danilewskyi*. 135

LIST OF FIGURES

Fig. 1-1. Life cycle of *Trypanosoma danilewskyi* 32

Fig. 2-1. Immunoblot of goldfish serum probed with anti-carp IgM hybridoma supernatant. 49

Fig. 3-1. Mortality, prevalence of infection, and course of infection in goldfish that were inoculated with *Trypanosoma danilewskyi*. 56

Fig. 3-2. Mortality, prevalence of infection, and course of infection, in naïve or rechallenged goldfish. 57

Fig. 4-1. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium supplemented with goldfish, carp, or tin foil barb serum. 79

Fig 4-2. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium supplemented with foetal bovine serum (FBS), goldfish serum (GFS), or FBS and GFS. 80

Fig. 4-3. *In vitro* growth of *Trypanosoma danilewskyi* cultured in serum-free medium or sera collected from infected (75 dpi) or naïve fish. 81

Fig. 4-4. *In vitro* growth of *Trypanosoma danilewskyi* cultured in sera collected from infected or naïve fish. 82

Fig 4-5. *In vitro* growth and morphological forms of *Trypanosoma danilewskyi* present in medium supplemented with goldfish serum. 83

Fig. 4-6. Wright's-stained smear of *Trypanosoma danilewskyi* cultured in goldfish serum. 84

Fig. 4-7. Wright's-stained smear of *Trypanosoma danilewskyi* cultured in goldfish serum. 85

Fig. 4-8. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium conditioned by 3 X 10^5, 10^5, 10^4, or no fish macrophages 86

Fig 4-9. *In vitro* growth of *Trypanosoma danilewskyi* cultured in control medium or medium conditioned by macrophages derived from uninfected or infected fish. 87

Fig 4-10. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium conditioned by macrophages derived from uninfected or infected fish... 88

Fig. 4-11. *In vitro* growth of *Trypanosoma danilewskyi* cultured in control medium or medium conditioned by human monocytes, U937. 89

Fig. 4-12. Protein concentration of undiluted control medium or medium conditioned by human monocytes, U937. 90

Fig. 4-13. *In vitro* growth of *Trypanosoma danilewskyi* cultured in control medium or medium conditioned by murine macrophages, P388D.1. 91

Fig. 4-14. Protein concentration of undiluted control medium or medium conditioned by fish fibroblasts, CCL71. 92

Fig. 5-1. Effect of intravenous administration of trypanosome excretory-secretory products to goldfish, prior to challenge with *Trypanosoma danilewskyi*. 136

Fig. 5-2. Effect of intramuscular administration of trypanosome excretory-secretory products to goldfish, prior to challenge with *Trypanosoma danilewskyi*. 137

Fig. 5-3. Effect of intraperitoneal administration of trypanosome excretory-secretory products to goldfish, prior to challenge with *Trypanosoma danilewskyi*. 138

Fig. 5-4. Effect of intraperitoneal administration of trypanosome excretory-secretory products/Freund's incomplete adjuvant, to goldfish, prior to challenge with *Trypanosoma danilewskyi* 139

Fig. 5-5. Effect of intraperitoneal administration of trypanosome excretory-secretory products/Freund's complete adjuvant to goldfish, prior to challenge with *Trypanosoma danilewskyi*. 140

Fig. 5-6. Immunoblot of *Trypanosoma danilewskyi* excretory-secretory products probed with serum from immune and normal fish.......... 141

Fig. 5-7. Infection dynamics of non-immunized goldfish (inoculated with *Trypanosoma danilewskyi* or uninfected) included in the trypanosome excretory-secretory product immunization experiment. 142

Fig. 5-8. Infection dynamics of non-immunized goldfish (inoculated with *Trypanosoma danilewskyi* or uninfected) included in the trypanosome lysate immunization experiment. 143

Fig. 5-9. Infection dynamics of non-immunized goldfish (inoculated with *Trypanosoma danilewskyi* or uninfected) included in the water-soluble molecule immunization experiment. 144

Fig. 5-10. Infection dynamics of non-immunized goldfish (inoculated with *Trypanosoma danilewskyi* or uninfected) included in the detergent-soluble molecule immunization experiment. 145

Fig. 5-11. Blastogenic response of peripheral blood lymphocytes isolated from immune or naïve fish that were incubated with trypanosome lysate 146

Fig. 5-12. Mortality, prevalence of infection, and course of infection in naïve goldfish that were immunized with immune or normal serum, prior to challenge with *Trypanosoma danilewskyi*. 147

LIST OF ABBREVIATIONS

AMP	adenosine monophosphate
ANOVA	analysis of variance
APRT	adenosine phosphoribosyltransferase
ATCC	American type culture collection
ATP	adenosine triphosphate
C3	Component of complement pathway
CCL71	goldfish fibroblast-like cell line
DMEM	Dulbecco's modified Eagle medium
dpi	days postinfection
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ES	excretory-secretory
FBS	foetal bovine serum
Fc	crystallizable fragment
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GFLM	goldfish leukocyte medium
GFS	goldfish serum
HEPES	$C_8H_{18}N_2O_4S$
Ig	immunoglobulin
kDa	kiloDalton

L4NHS	undefined medium developed by [Rouskova, 1983 #324]
L929	murine fibroblast-like cell line
N/A	not applicable
NCC	nonspecific cytotoxic cell
NK	natural killer
N.D.	not done
N.S.	not significant
P388D.1	murine macrophage/monocyte-like cell line
PBL	peripheral blood leukocyte
PBS	phosphate-buffered saline
PBSN	phosphate-buffered saline-sodium azide
PSG	phosphate saline-glucose
RPMI 1640	Rose Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	one standard error of the mean
SNB9	diphasic blood agar medium
TBS	tris-buffered saline
TTBS	tris-buffered saline containing Tween 20
T_{H1}	Helper T-cell population (induces T-cell response)
T_{H2}	Helper T-cell population (induces B-cell response)
TNF	tumor necrosis factor
U	International unit
U937	human monocyte-like cell line

VSG	variant surface glycoprotein
WSM	water-soluble molecules

CHAPTER I

INTRODUCTION

The fresh-water haemoflagellate *Trypanosoma danilewskyi* (Laveran and Mesnil, 1904) occurs naturally in common carp, *Cyprinus carpio*, crucian carp, *Carassius auratus gibelio*, tench, *Tinca tinca*, and eel, *Anguilla* spp. [185]. The prevalence of infection may approach 100% in densely populated fish cultures [215]. Depending on the species, the severity of infection can range from subclinical to severe [185, 298]. Clinical disease in experimentally infected goldfish, *Carassius auratus*, frequently includes anorexia, anemia, and death. A high number of goldfish (up to 80%) die from parasitemia if inoculated with a large number of trypanosomes. However, this mortality could be reduced considerably if the goldfish were injected with a lower number of flagellates [140, 183, 283, 298].

Although there are 190 species of fish trypanosomes (see [186]), little is known about the factors that stimulate division and morphogenesis of these parasites. The first report of *in vitro* cultivation of *T. danilewskyi* was by Thomson in 1908 [273]. He reported that when infected fish blood was inoculated into blood agar, containing ox flesh and defibrinated rabbit blood, the haemoflagellates survived for 43 days. Smolikova *et al.* [251] showed that proliferation may be the result of a vital reaction of the parasite to a compound in fish, because culture medium without the presence of living cells failed to support their growth. This was supported by the observation that medium containing mammalian blood, or medium supplemented with foetal bovine serum (FBS) was unsuitable for culturing *T. danilewskyi* [35, 141, 216]. Other medium, such as L4NHS containing rabbit serum and erythrocyte lysate from rabbit blood supported long-term cultivation of the parasite [233]. In general, these cultivation protocols require complex semi-defined medium. For future immunological and biochemical investigations, it would be advantageous to have a defined medium that supports long-term propagation.

Such a medium has been published [284], although it was not reproducible by our laboratory [35].

The concept of protective immunity to *T. danilewskyi* stems from four observations: 1) protection was passively transferred by immune plasma [298] or immunoglobulin (Ig) M purified from immune serum [215]; 2) injection of a corticosteroid into recovered fish caused a reoccurrence of a patent infection in some fish [140]; 3) fish surviving the primary infection were resistant to reinfection [140, 283, 298]; and 4) trypanosome infection was accompanied by seroconversion in experimentally infected carp, using an enzyme-linked immunosorbent assay (ELISA) with antigen extract (parasite lysate) [215]. Altogether, these results suggest that resistance was associated with humoral immunity and memory cells. However, this does not necessarily imply that elimination of the primary infection was solely due to recognition of antigen by antibodies, as Li and Woo [176] reported that non-immunological factors (i.e., anorexia and plasma protein) affected the multiplication rate of kinetoplastids. Further, very little research has been done to elucidate the involvement of nonspecific or cell-mediated immune mechanisms of resistance to *T. danilewskyi*.

In general, the purpose of my doctoral research was to provide a better understanding of the complex relationship between trypanosomes and their vertebrate hosts. This was accomplished by: 1) determining growth requirements of *T. danilewskyi in vitro*; and 2) examining components of the host-parasite relationship that influences the susceptibility of goldfish.

The remainder of this chapter reviews the literature pertaining to the general biological aspects of *T. danilewskyi* and immunobiology of parasitic protozoa of fish. Additionally, it contains an overview of metabolic processes and substances that potentiate the growth of extracellular trypanosomes. The second chapter provides a detailed description of the general material and methods that were used. The course of *T. danilewskyi* infection (first and second exposure) is described in Chapter III. Chapter IV presents cultivation of

T. danilewskyi in vitro, using homologous and heterologous serum. Also, this chapter describes cultivation of *T. danilewskyi* in soluble cell products obtained from piscine or mammalian sources. Chapter V presents the results of immunization experiments that screened different types of antigenic preparations. In these experiments, several parameters (i.e., parasitemia, prevalence of infection, red cell volume, and the humoral response) were examined to determine the efficacy of the immunization strategy. The final section (Chapter VI) is a general discussion. Because of the diverse nature of my thesis, the summary of results and the general discussion will be integrated with recommendations of future directions of research.

Objectives

1) Determine the course of *T. danilewskyi* infection (first and second exposure) in goldfish that were housed in the stated conditions.

2) Define the conditions that are necessary for trypanosome growth. More specifically,

   a) Determine whether *T. danilewskyi* can be cultivated in medium supplemented with fish serum (goldfish, carp, or tin foil barb, *Puntius schwanenfeldi*), and to compare the *in vitro* growth curves in serum-free medium or medium containing mammalian serum.

   b) Conduct a morphometric study of cultured *T. danilewskyi* to determine the proportion of life-cycle forms present in stock cultures, maintained in goldfish serum (GFS).

   c) Study the association between host cell (macrophage and fibroblast) products and the growth of parasites. This includes determining whether the: i) products of macrophages, isolated from infected or uninfected fish, affect parasite growth; ii) activity of cell-conditioned medium was specific to species or cell-type; and iii) *in vitro* growth of trypanosomes correlates with the protein concentration of the cell culture supernatants.

3) Determine the efficacy of immunizing of goldfish against *T. danilewskyi.* Parameters used to assess the efficacy of immunization included: mortality, prevalence of infection, abundance of parasites, red cell volume, and the presence of parasite-specific antibodies.

a) Determine the effects of antigen preparations (sodium perchlorate-killed parasites, parasite lysate, water-soluble molecules, detergent-solubilized molecules, and excretory-secretory (ES) products) and inoculum route on the susceptibility of goldfish to *T. danilewskyi.*

b) Conduct a pilot study to give corroborative evidence of passive transfer of immunity to naive fish by inoculation of serum from an immune donor.

LITERATURE REVIEW

History

Trypanosoma danilewskyi is a protozoan parasite of the phylum Sarcomastigophora, class Zoomastigophorea, and family Trypanosomatidae that was named and identified in 1904, by Laveran and Mesnil. Other species described under different names (*T. carassii*, *T. tincae*, and *T. wincheniense*) are believed to be conspecific [186]. Phylogenetic evaluations inferred that this organism evolved from an amphibian trypanosome *(Trypanosoma rotatorium*) that was paraphyletic with *Trypanosoma brucei* representing the earliest diverging lineage [98, 192].

Under experimental conditions, over 16 different species of fish (i.e., carp, brown bullhead, *Ictalarus nebulosus*, European eel, *Anguilla anguilla*, giant danio, *Danio* sp., rainbow darter, *Etheostoma caeruleum*, white sucker, *Catostomus commersoni*, etc.) were susceptible to *T. danilewskyi* [294, 303]. In nature, *T. danilewskyi* has been found in France [190], Russia [4], Poland [294], India [225], and the Black Sea District of the Ponto-Aralo-Caspian Province [73].

Most piscine trypanosomes are regarded as nonpathogenic haemoflagellates [307] of no economical or veterinary importance. However, the marked increase in aquaculture in the last few decades [196, 234, 304] has heightened the interest in diagnosis, treatment, and prevention of trypanosomiasis. For example, in densely populated cultures of tench or carp, the prevalence of *T. danilewskyi* infection may approach 100% [215]. Further, an aquaculture farm in Taiwan reported an outbreak of *Trypanosoma* spp. that caused clinical signs (i.e., anorexia, anemia, and splenomegaly) in 10% of the fish [135].

Life cycle

The life cycle of *T. danilewskyi* is illustrated in Fig. 1-1. Infection of the vertebrate host occurs when metacyclic trypomastigotes are transmitted during a blood meal of an infected leech (i.e., *Hemiclepsis marginata* or *Piscicola geometra*). After 2 to 9 days, *T. danilewskyi* is detected in the peripheral blood of the fish [186]. After developing into bloodstream forms, a rapid increase in the number of parasites occurs in the blood during the early days of the infection [230]. Although sexual reproduction has not been documented for *T. danilewskyi*, there is evidence this occurs in other trypanosomatids [144, 262, 263]. The asexual multiplication process of bloodstream-forms has been reported to occur in four stages [299]. The first phase of multiplication is the production of a flagellum and subsequent division of the kinetoplast. Thereafter, formation of new cytoplasm occurs at the posterior end. A well-formed posterior end with a weak undulating membrane defines the third stage of division. As well, nuclear division commences with migration of one of the nuclei past the two kinetoplasts. At the final stage of the multiplication process, *T. danilewskyi* resembles two fully formed parasites joined at their posterior end (i.e., symmetrogenic division). Division is completed by a transverse constriction at a point between the kinetoplasts. Eventually, the bloodstream-forms proceed into a chronic or stationary phase of the infection, in which there is no division [186, 230].

Along with the blood of an infected fish, leeches take up bloodstream-forms into the crop. Comprehensive morphometric descriptions of the development of *T. danilewskyi* in the leech were described previously [225, 230]. In brief, within the first 3 hr of the blood meal, the trypanosomes decrease in size with a concentration of the cytoplasmic contents. The posterior two-thirds of the parasite expand and the undulating membrane and the free flagellum shorten. After 1 day, stumpy forms with the kinetoplast approaching the nucleus are present in the viscous blood. Only a few stumpy-forms undergo equal binary division. The parasites then assume a crithidial-form, which seems to be able to divide continuously, so that, the crop is teeming with large numbers of tadpole-shaped flagellates with the kinetoplast located closely by the nucleus. After 4 days, a pleomorphic population is present in the crop of the leech. However, leptomonad-forms (elongated individuals with the nucleus situated toward the posterior end and the kinetoplast in the anterior half) are also observed at this time. After the fifth to seventh day, slender-forms arise. Thereafter, the morphological development comes to a close. Thin attenuated metacyclic-forms with elongated nuclei pass from the crop to the proboscis and are seen lying in the sheath. Metacyclic trypomastigotes can persist in the sheath for more than 2 mo. If the leech is unable to attain another blood meal, the parasites in the proboscis sheath degenerate and die. However, other metacyclic-forms continuously migrate forward from the crop.

Pathogenesis

Piscine trypanosomiasis were considered to be innocuous in feral fish populations, as the abundance of parasites in natural infections were generally low [30, 239]. However, few researchers have explored the long-term effects of *T. danilewskyi*. As with other fish-parasite systems, it is conceivable that a concurrent stressor can impair the health of fish in inconspicuous ways, such as undermining their ability to reproduce and resist disease. For example, in laboratory studies Khan (1988) demonstrated that 53% of gadoid fish parasitized by *Trypanosoma mumanensis* and a haematophagous copepod

(*Lernaeocera branchialis*) died, whereas 24% with the trypanosome or 12% with the copepod. Moreover, those fish with concurrent infections were emaciated to the extent that survival in nature was thought to be unlikely [155].

Trypanosoma danilewskyi caused morbidity and mortality in experimentally infected fish [138, 185, 298]. Pathogenic potential of *T. danilewskyi* was associated with the infection intensity. When inoculated with a large number of trypanosomes, a high number of goldfish (up to 80%) died. However, this mortality was reduced considerably when goldfish were injected with a lower number of flagellates [140, 183, 283, 298].

In experimentally infected goldfish, *T. danilewskyi* infection causes anorexia [138] and severe histopathological changes in the haemopoietic organs [81]. For instance, minute foci of necrosis and excessive disintegration of red blood cells were observed in the spleen. The kidneys had a marked increase in the number of haemoblasts and plasma cells with disintegration of red blood cells and dystrophic changes of the tubule epithelium. Also, dystrophic changes of hepatocytes and swelling of endothelial cells of hepatic sinuses appeared at 7 days postinfection (dpi) [81]. Clearly, the damage of the haemopoietic tissue corresponded with the anemic condition that was often characterized by watery blood, pale-colored gills [230], and a significant decline of the packed red cell volume [139, 283]. At present, the physiological changes in the blood of trypanosome-infected fish are poorly understood. Tandon and Joshi [265] reported that two species of fresh-water fish trypanosomes caused: 1) leukocyte (neutrophils, macrophages, eosinophils, basophils, and monocytes) counts to rise; 2) thrombocytes and large lymphocytes to reduce (25 to 50% of their number in a healthy fish); and 3) a decrease in total erythrocyte counts, with a marked decline in their haemoglobin content.

Control measures/therapeutics

In animal husbandry, measures to prevent the introduction or onset of disease are always the most effective, cost-efficient, and long lasting [196]. Conceivably, successful control of *T. danilewskyi* infection would include: 1) prevent the introduction of trypanosomes ; 2) maintenance of good environmental conditions (i.e., temperature, water chemistry, pH, etc.); 3) chemotherapy; and 4) immunization.

In nature, leeches with metacyclic trypanosomes transmit *T. danilewskyi* [27, 303]. Consequently, eliminating the intermediate host may prevent trypanosome infections. Removing fish from infested water may not be a feasible measure to control leeches, as *Piscicola* and *Hemiclepsis* survived for a prolonged period (3 and 10 mo, respectively) in water without fish. Draining the water source killed leeches, because they can endure dry conditions only for a short time. However, draining cannot destroy the cocoons. For this reason, additional measures (i.e., calcium oxide treatment) need to be taken to limit the moist depressions [239]. Controlling bath solutions of natural molluscicides (i.e., aridanin, aridan, and endod), niclosamide [105], and copper chloride [25] were shown to be successful for leech control. Notwithstanding, use of such chemicals should be used with discretion, as non-target organisms may be sensitive to hirudicidal agents [105]. Biological control may serve as a feasible alternative to pesticides, as Raut and Saha [227] reported that egg-bearing *H. marginata* were susceptible to the attack by the fungal parasite *Anguillospora* sp. Further, *H. marginata* has a number of natural enemies, as fish and semi-terrestrial leeches readily serve as predators [230].

Information pertaining to the chemotherapeutic removal of trypanosomes and related kinetoplastids from the blood of fish is limited. Tryparsamide [275], calcium cyanamide, and calcium oxide [127] have been suggested for the treatment of *Trypanosoma*, *Trypanoplasma*, or *Cryptobia* species. Administration of 50 g of metronidazole per kg of fish weight daily (for 6 days) has been an effective treatment against these parasitic diseases [239]. *Cryptobia salmositica* (synonym *Trypanoplasma salmositica*) infections in rainbow trout, *Oncorhynchus mykiss*, have been reduced by prophylactic administration and booster injections of 0.01 or 0.1 mg kg^{-1} isometamidium chloride [15]. When applied to *Cryptobia cyprini in vitro*, methylene blue and gentian violet stain exerted an anti-cryptobial effect. Further, administration of these stains to *Cryptobia*-

infected fish, by immersion bath or a cannula into the digestive tract, eliminated or substantially reduced the vitality of the parasite [125].

At present, no preventative vaccine against piscine kinetoplastids is commercially available. Nevertheless, a number of researchers have described host-parasite systems that demonstrate the potential of immunization strategies. The attenuation of *C. salmositica* has been described. Immunization of rainbow trout, brook charr, *Salvelinus fontinalis,* or chinook salmon, *Oncorhynchus tshawytscha,* with this virulent strain resulted in low parasitemias and protection against subsequent challenge with the avirulent parasite [12, 29, 177, 178, 254, 306]. Acquired immunity can be passively transferred to naïve fish with the plasma from convalescent fish [94, 298]. For instance, Woo [298] reported that no parasites were found in the blood of goldfish administered homologous immune plasma and subsequently challenged with 3000 *T. danilewskyi*. Similar to that observed in the passive transfer of homologous immune plasma, the efficacy of a mouse monoclonal antibody (directed against surface molecules of *C. salmositica*) in infected rainbow trout was reported [94, 302]. This study demonstrated that parasitemias in fish, infected with *C. salmositica*, were reduced (>50%) after administration of a monoclonal antibody directed against a 200 kDa molecule present on the parasite surface. As demonstrated *in vitro*, it was possible that this murine antibody reduced parasitemia by agglutination of parasites and subsequent phagocytosis [91]. However, the possible role of nonspecific factors cannot be excluded, as an isotype control was omitted from the *in vivo* experiment. Nevertheless, these studies suggest that a potential development of immunochemotherapy for the control of blood-borne diseases of fish may be feasible.

Immunobiology of parasitic fish protozoa

The immunobiological relationship between *T. danilewskyi* and its host is poorly understood. For this reason, a treatise of the interactions between parasitic protozoa and the fish immune system is presented. Such an account will provide a better understanding of future research that is required for the development of immunomodulation strategies. A brief summary of the biology of these protozoa is provided in Table 1-1.

Humoral Immunity. Although the nature of acquired immunity to fish protozoa is not clear, it resulted in the appearance of serum antibodies that were parasite specific [52, 59, 63, 72, 132, 147, 148, 159, 173, 215, 250, 280, 289, 300, 305]. Similarly, an intestinal coccidian of fish evoked a detectable increase in the number of Ig+ lymphocytes within the pronephros and the intestine [255, 256]. In many of these studies, the peak of the humoral response coincided with the decline in parasitemia. Notwithstanding, the presence of a humoral response does not necessarily infer its role in clearing the infection. For example, Kim *et al.* [159] concluded that acclimation temperature and not the resulting increase in parasite-specific antibodies facilitated protection against a microsporidian parasite. With this concept in mind, the remainder of this section will present the function of humoral immunity in prevention or elimination of protozoan diseases.

Passive transfer of protective immunity from mother to young has been illustrated in fish, as high molecular weight agglutinating antibodies (669 kDa) were detected in the eggs of immunized plaice, *Pleuronectes platessa* [38]. Passive transfer of antibodies to fry was also demonstrated in tilapias, *Oreochromis aureus*, vaccinated against *Ichthyophthirius multifiliis* [247]. The latter study demonstrated that the protection against *I. multifiliis* infection was attained directly from the mother via the eggs and indirectly from the mucous membranes of the mother's mouth during the brooding period. Hines and Spira [132] postulated another indirect form of passive transfer. Namely, serum proteins on the exterior surface of an immune fish diffused into the small volume of water surrounding the fish and subsequently neutralized pathogens before they came into contact with the fish.

Under experimental conditions, passive transfer of immunity has been successful using immune plasma or monoclonal antibodies directed against parasite surface membranes. As demonstrated in haemoflagellate systems, inoculation of plasma from recovered fish conferred protection against the parasite in naïve recipients [148, 298]. The presumed role of antibodies in these experiments was confirmed using purified IgM, derived from serum of carp recovered from a *T. danilewskyi* infection [215].

When administered therapeutically, monoclonal antibodies (directed against *C. salmositica* or *I. multifiliis* surface antigens) conferred protection to naïve rainbow trout and channel catfish, *Ictalurus punctatus*, respectively [60, 94, 180]. In one case, parasitic infection was controlled by administration of monoclonal antibody subfragments (i.e., fragments of antigen binding ($F(ab)_2$)) [60]. Taken together, these studies demonstrated that the efficacy of passive immunization was associated with the: 1) parasite strain; 2) isotype of the monoclonal antibody; and 3) concentration of monoclonal antibody administered.

The infectivity of fish protozoa can be neutralized by solutions containing anti-parasite antibodies [91, 289, 298]. Successful neutralization of parasite infectivity appeared to be contingent on several factors. For instance, juvenile rainbow trout responded more favorably than adult fish [91]. Secondly, the infectivity of cultured *C. salmositica* was neutralized by incubation in immune plasma, whereas no neutralization was observed using parasites isolated from the blood of infected fish [148]. The efficacy of neutralization was also influenced by temperature and length of incubation [298]. The mechanism of neutralization could occur by numerous pathways. Jones and Woo [148] suggested that neutralization by protective antibodies involves recognition and binding of antigenic sites on the parasite. Further, antibodies may affect metabolic processes, as incubation of *C. salmositica* in protective antibodies hindered multiplication and eventually killed the parasite [91].

Aggregation of insoluble antigen caused by the reaction with multivalent antibodies (i.e., agglutination) has been described in a few parasite systems [59, 91, 148, 215]. Presumably, parasite agglutination resulted from cross-linking of protective antibodies to cell surface molecules. For this highly motile protozoan, it was possible that immobilization of the organism preceded agglutination. Immobilization of free-swimming stages of *I. multifiliis* has been demonstrated in studies using immune fish serum or mucus [132, 173, 248, 280], rabbit antiserum prepared against ciliary antigens [59, 72], and murine monoclonal antibodies directed against surface membrane proteins [60]. It was unlikely that the forces generated during movement were overcome by antigen-antibody interactions, as the cilia of *I. multifiliis* continued to beat in the presence of immune serum [60]. The latter authors observed that agglutination was accompanied by the production of mucus and proposed that these secretions entrap the cells or interrupt the ciliary beat. The parasite aggregate could then facilitate antibody-mediated effector mechanisms.

Anti-parasite antibodies conferred protection by altering the behavior of the parasite. *Ichthyophthirius multifiliis* rapidly exited the epidermis of immune fish or those passively immunized with a monoclonal antibody [60, 279]. Emerging parasites had detectable antibodies on their surface [60]. Although parasites expelled from an immune fish could invade another fish, its infectivity was lower than those artificially expelled from naïve fish [279].

While numerous studies demonstrated that parasite infection induced the production of antibodies, it is also important to note that some fish protozoa suppressed humoral immunity. Inoculation of *Glugea* spp. or disrupted spores caused a significant decrease in IgM [167-170, 223]. The magnitude of suppression was proportionate to the number of spores in the inoculum [168]. Similarly, repeated inoculations caused a further decrease in the presence of IgM [167, 168]. In this parasite system, immunosuppression appeared to be independent of antigenic competition, as inoculation of antigen (i.e., red blood cells) resulted in a decrease of IgM, but not as low as in those fish inoculated with the parasite alone [167, 168, 170]. Collectively, these workers suggested that a soluble parasite molecule (113 kDa) stimulated leukocytes to release mediators (i.e., prostaglandin) that suppressed the initiation of the immune response. While this hypothesis was supported by experiments using indomethacin (suppressor of prostaglandin), it does not exclude the possibility that parasite molecules could breakdown IgM or decrease its half-life [167, 168, 223].

Although not studied as extensively, immunosuppression has been documented in haemoflagellate systems. These studies showed that infection with *C. salmositica* produced the suppression of the humoral response against red blood cells [152, 289]. *Cryptobia salmositica* also caused suppression of the antibody response against *Yersina ruckeri*, resulting in an increase in mortality in those fish given a concurrent infection [152].

Complement. The complement cascade consists of a system of serum proteins that acts in conjunction with the antibody and cell-mediated immune response to provide and enhance specific effector functions (reviewed by [5, 107, 236]). Although no fish species has been found whose complement system was biochemically equivalent to that of mammals, a number of comparable macromolecules have been described in nurse shark, *Ginglymotoma cirratum*, tuna, *Thunnus* spp., carp, and rainbow trout [204, 236]. Binding of the first component of the complement pathway to the Fc (crystallizable fragment) portion of an antigen-antibody complex activates complement. A variety of substances (i.e., microbes, bacterial endotoxin, fungal polysaccharide, animal venom, etc.) trigger the alternative complement pathway. Activation of these pathways result in a cascade of proteolytic reactions that produce a membrane attack complex, resulting in lysis of the target cell. At the same time, proteolytic fragments released during the activation process promote the defense response by acting as an opsonin, chemotaxin, or anaphylatoxin. Of the numerous functions of complement, the *in vitro* immune lysis test has been used most extensively to study protozoan diseases of fish. In general, this processes occurs quickly, requiring only 30 to 60 min for complete lysis (approximately 500 organisms in 0.025 ml plasma) [290]. Lysis of *T. danilewskyi* by the alternate complement pathway has not been documented. Nonetheless, it seems unlikely that this was a protective mechanism in susceptible fish, as the surface coat was postulated to be negatively charged. These charges may provide a protective layer and render the parasite more resistant to lysis [182].

For protozoa of fish, the role of the classical complement pathway has primarily been characterized using *C. salmositica*. Complement fixing antibodies were detected in the plasma of *Cryptobia*-infected rainbow trout 5 wk after infection. After the peak of antibody titres at 10 wk, the titres decreased markedly. At 16 wk postinfection there were almost no detectable complement fixing antibodies. Recovered fish had a rapid secondary response 1wk after homologous challenge [93].

As a means of controlling flagellate infections, numerous workers have reported environmental and experimental manipulations that affect the production or efficacy of complement fixing antibodies. Collectively, field observations and results from the *in vitro* immune lysis test suggested that elevated water temperatures (20 to 24 °C) protected sockeye salmon, *Oncorhynchus nerka*, and summer flounder, *Paralichtys dentatus*, against kinetoplastid infections [42, 259]. In addition to temperature, production of trypanocidal antibodies was also dependent on the nutritional status and social position of the fish [27, 28]. In rainbow trout, complement-fixing antibodies were adversely affected by the salinity of the water. For instance, protection was delayed in fish maintained in sea water and the antibody titre was significantly lower than in fish maintained in fresh water [178]. A treatment trial with the drug isometamidium chloride resulted in the production of complement fixing antibodies that was higher than in untreated controls [15]. Lastly, several publications reported that vaccination strategies with an avirulent *C. salmositica* strain resulted in a rapid increase of complement fixing antibodies that lysed the parasite *in vitro* [12, 13, 93, 177].

Studies using heat inactivation, chelating agents, carageenan, or zymosan determined that the alternative complement pathway facilitates innate resistance against fish protozoa [13, 42, 44, 102, 165, 290]. Interestingly, resistance varied between fish species [44, 102, 290] and within a species [13, 42, 101, 102]. Using laboratory bred brook charr, Forward *et al.* [101] determined that resistance against *C. salmositica* (via the alternative pathway) was attributed to a single Mendelian locus [101].

Although complement-mediated cytolysis has been repeatedly documented, it appears as though numerous response mechanisms mediate infection. In brief, passive transfer with a monoclonal antibody, directed against a surface antigen of *C. salmositica*, conferred protection, although only 30% of the fish had complement fixing antibodies [94]. Thymectomized (2 mo) rainbow trout had reduced the detectable protective antibodies, while parasitemia in these fish was significantly lower than in intact fish [93]. A study investigating the effect of serum of blue tilapia, *Tilapia aurea*, on the infectivity of a parasitic dinoflagellate demonstrated that normal serum (1.25%) inhibited the ability of the parasite to infect host cells [165]. This anti-parasitic effect was partially attributed to complement, as heat-inactivation, zymosan, or carageenan neutralized the activity. However, other factors contributed to the anti-parasitic effect observed in naïve blue tilapia, as the activity observed in serum was partially resistant to heating. Buchmann *et al.* [50] reported that normal serum from rainbow trout immobilized the fresh water ciliate, *I. multifiliis*. In contrast, decomplementized serum (44°C, 20 min) showed no immobilization. The opsonic effects of complement and/or antibodies have not been directly studied in blood-borne parasite systems. Nevertheless, a few workers have reported phagocytic activity against *T. danilewskyi* [81], *Trypanoplasma bullocki* [51, 259], and *C. salmositica* [177].

Phagocytes. Phagocytosis, the ingestion of particles by a cell, has been conserved in evolution and is probably the main defense of most of the animal world. In a process called opsonization, antibodies and complement are known to enhance phagocytic activity (reviewed by [107, 244]). Relevant surface receptors (i.e., Fc and C3) have been reported on fish phagocytes (reviewed by [244]). The mechanism of opsonization was presumably similar to that described in mammalian systems. Briefly, antibodies or complement bound to the foreign particle were available to react with the receptors on the surface of the phagocyte. As the phagocyte reacted with the receptors, the cell membrane moved over the surface and engulfed the particle, essentially zippering the membrane closed around the particle (reviewed by [107]).

Phagocytosis of *T. danilewskyi* [81], *C. salmositica* [297], *T. bullocki* [51, 259], myxosporeans [217, 249] and microsporidians [80, 82, 224] have been observed in histological sections or peritoneal fluid taken from parasitized fish. Inasmuch as phagocytosis can occur in the presence or absence of opsonins, the role of opsonic activity in these parasite systems was uncertain. Using an *in vitro* assay, a more recent study has given evidence for opsonization of *C. salmositica*. Notwithstanding, the number of macrophages with engulfed *C. salmositica* was seemingly low in immune and nonimmune serum samples, 2.8% and 1.3%, respectively [177].

Microsporidian parasites were able to manipulate phagocytosis and non-specific responses [174]. For example, the presence of complement or antibodies greatly enhanced phagocytosis of inert particles, but phagocytosis of microsporidians (*Glugea* sp. or *Tetramicra brevifilum)* was not affected by either. In this system, the authors postulated that the presence of microsporidian spores led to degradation or modification of sugar-borne receptors that were necessary for engulfment.

Leiro *et al.* [175] reported that turbot, *Scophthalmus maximus*, spleen- and pronephros-resident phagocytes exposed to viable microsporidian spores produced significantly less superoxide than cells exposed to non-viable spores [175].

T-cell-mediated responses. Information of the T-cell (thymus –dependent lymphocyte) immune response in protozoan-infected fish is very sparse. In histological studies the role of cell-mediated immunity has been inferred by the infiltration of leukocytes into infected tissues [217, 249]. Additional evidence of T-cell-mediated responses included the delayed-type hypersensitivity reaction [92, 248, 271], macrophage migration inhibition assay [248, 271], and adoptive transfer of leukocytes from immune to naïve fish [148].

Delayed hypersensitivity reactions were mediated by T-cells both through the release of lymphokines and through exertion of direct cytotoxicity. Initiation of the reaction was by antigen deposited on the skin (see [103, 107]). In *C. salmositica* and *I. multifiliis* systems, the delayed-type hypersensitivity reactions were induced by natural infection or vaccination with an avirulent strain [92, 248, 271]. Intradermal inoculation of antigen resulted in induration at the injection site characterized histologically by infiltration of mononuclear leukocytes. In one study, the magnitude of the hypersensitivity reaction was markedly affected by the diet of the fish [271].

Cell-mediated immunity was expressed *in vitro* by the inhibition of macrophage migration. When the respective parasite antigen was incubated with sensitized T-cells and macrophages, the lymphocytes produced migration inhibitory factor that resulted in temporary inhibition of migration of localized macrophages. This phenomenon was demonstrated using the kidney cells of goldfish and rainbow trout sensitized against *I. multifiliis* and *C. salmositica*, respectively [248, 271].

In fish, the T-cell subsets and their phenotypic markers are not defined as well as in mammalian systems [58]. Nevertheless, there was suggestive evidence of suppressor-like populations. For instance, *C. salmositica* infection in rainbow trout inoculated with thymocytes from naïve fish was significantly higher than in control fish [92]. The latter authors attributed this observation to a suppressor cell population that diminished the protective mechanisms or potentiated parasite multiplication. An alternative explanation for this observation could be that inoculation of lymphocytes into recovered fish caused immunosuppression (i.e., overwhelming antigenic challenge).

Nonspecific cytotoxic cells. Of the cellular responses elicited in fish, nonspecific cytotoxic cells (NCC) have been studied most extensively. Although NCC were found in the spleen and peripheral blood of normal fish, the highest cytolytic activity was found in the head kidney [85]. Within the anterior kidney, at least four different-sized populations of NCC were elucidated [90]. Morphologically, NCC were described to be monocyte-like (i.e., reniform nucleus and a low nucleus/cytoplasm ratio) [84]. However, they shared many functional properties with mammalian natural killer (NK) cells. For example, both NCC and NK cells: 1) are not phagocytic; 2) are suppressed by Igs present in autologous sera [86]; 3) require cell-to-cell contact with target cells [85]; and 4) target a broad spectrum of susceptible cells [112, 142, 172].

With regard to the cellular defenses against parasites, Graves *et al.* [113] reported that *I. multifiliis*-infected fish demonstrated a shift of NCC activity from the head kidney to the peripheral blood. Further, the NCC within the peripheral blood of moribund fish have an increased killing capacity and a 36-fold increase in target cell affinity than those cells from naïve fish. Agglutination or deciliation of the protozoan *Tetrahymena pyriformis* facilitates binding of the NCC to the parasite [112]. Conjugation between effector and target cell was mediated by a dimeric molecule (38 kDa and 41 kDa molecule) present on NCC [87] and resulted in: 1) increased DNA replication in NCC; 2) *in vitro* proliferation of NCC [88]; and 3) significant killing of parasites after 10 hr [112].

A 46-50 kDa target antigen (NKtag) of *T. pyriformis,* involved in target cell recognition by NCC has been identified [142, 172]. Mapping of this protein determined that the binding site of the antigenic determinant consisted of a minimum of seven to nine amino acids in the N-terminal region [89]. Further, amino acid sequences indicated that the NKtag was a novel protein that shared some sequence homology with glycolytic enzymes [142].

Antigens of *Trypanosoma* spp.

Major surface antigens. For many protozoa, antigenic variation occurs during parasite ontogeny (reviewed by [242]). That is to say, the various life cycle forms may be antigenically distinct. By the time the host evoked an immune response against the infective form, the trypanosome has differentiated into the bloodstream form.

The bloodstream form of African trypanosomes (*T. brucei* and *T. rhodesiense*) caused chronic disease by varying the coat that covered the trypanosomal surface, called the variant surface glycoprotein (VSG) (reviewed by [107, 163, 241, 242]). The antibody response and the associated effector functions eliminated most of the parasites (~ 99%). The surviving organisms, which have an antigenically distinct VSG, escaped the immune response and proliferated to produce a successive parasitemia. A single trypanosome has the ability to spontaneously produce over a hundred different variants. The structure of the variant glycoprotein has been described as a 50 kDa glycosylated peptide that was anchored into the plasma membrane by a linkage to phosphatidylinositol-containing glycolipid. The release of the surface glycoprotein from the trypanosome was facilitated by a VSG lipase [242].

At present, the literature suggests that the bloodstream forms of fish trypanosomes do not produce different major surface antigens. Assessment of the primary infection supported this hypothesis, as recrudescent waves of parasitemia were not observed (see Chapter III). Overath *et al.* [215] demonstrated the lack of antigenic variation by cross-protection studies. Namely, fish that had recovered from an infection with clone K1 were resistant against a challenge with parasite lines derived from chronically infected carp. Also, immunoblotting of homologous and heterologous clones of *Trypanosoma phaleri* against immune sera determined that these strains were antigenically similar [150].

Ultrastructural examination of *T. danilewskyi* illustrated the presence of a fuzzy carbohydrate-rich surface coat, similar to that described for *Trypanosoma cruzi* [216]. Just recently, Lischke *et al.* [182] reported that the surface coat of *T. danilewskyi* bloodstream forms contained mucin-like glycoproteins (~6 X 10^6 molecules $cell^{-1}$) that were anchored in the plasma membrane by glycosylphosphatidylinositol residues. Further, they demonstrated that serum from an immune fish recognized these molecules and that they do not undergo antigenic variation.

Parasite-released products. Proteins secreted by eukaryotes are produced on the ribosomes of the rough endoplasmic reticulum. After passing into the lumen of the endoplasmic reticulum, proteins are transported by vesicles to the Golgi apparatus. Vesicles to the cytosol transport molecules modified in the Golgi apparatus. During exocytosis the vesicle membrane is incorporated into the plasma membrane [5]. Ultrastructural examination of mammalian trypanosomes revealed that the majority of the exocytotic and endocytotic activity occurred within the flagellar pocket (reviewed by [288]).

While many excreted molecules were by-products of metabolic processes, others functioned in the evasion of defense mechanisms. For example: 1) excreted factors allowed *T. cruzi* to evaded complement-mediated lysis by interfering with the formation [99, 145, 146] and accelerating the intrinsic decay of the alternative pathway C3 convertase [145, 146]; and 2) excreted/secreted lysins caused direct lysis of red blood cells [139].

Numerous studies have demonstrated the protective value of products released from mammalian trypanosomes. Rodents immunized with ES products and challenged with trypomastigotes resulted in a marked reduction in parasitemia and mortality [56, 261]. Further, a recombinant ES molecule (24 kDa) or a 15 amino acid peptide (109-124), derived from the primary sequence of the 24 kDa polypeptide, had the capacity to induce protection against a lethal *T. cruzi* infection [260, 261].

Induction of an antibody response by trypanosome-released products has been determined [56, 119, 214, 260, 261]. In fish, a 200 kDa glycoprotein, an ES product of *C. salmositica*, was detected by antiserum collected from recovered trout [95]. In humans, sera obtained from patients with acute *T. cruzi* infection reacted with a family of 150-200 kDa bands and strongly with a pair of 45-55 kDa bands [143]. Conversely, sera from patients with chronic *T. cruzi* infection recognized 160 [143, 213], 130, and 80-110 kDa bands of ES antigens [213]. It appeared that there were common antigenic structures of these ES molecules and surface antigens, as: 1) monoclonal antibody directed against a 85 kDa surface protein immunoprecipitated with ES molecules of a similar weight; and 2) incubation of immune serum with ES antigens inhibited further binding of trypomastigote surface antigen to specific antibodies [213]. These observations were further supported by Goncalves *et al.* [108] who demonstrated that *T. cruzi* actively shed surface antigens (main cluster in the 70 to 110 kDa region) with plasma membrane-derived vesicles.

In addition to the induction of a specific antibody response, Taibi *et al.* [260] reported the protective activity of ES antigens to be associated with cell-mediated immunity. Splenic T-cells from chronically infected mice proliferate after *in vitro* stimulation with a 24 kDa recombinant polypeptide. Further, the cytokine profile of experimentally infected or immunized mice demonstrated that splenic cells elicit a T_{H1} response (secretion of interleukin-two or interferon-gamma, or both).

Metabolism and growth of trypanosomes

Nutrient acquisition. Similar to all cells, protozoa are surrounded by a trilaminar unit membrane (reviewed by [186, 194]). Nutrient acquisition across the membrane occurs by diverse mechanisms. Passage of nutrients across the outer membrane occurs by passive processes (i.e., diffusion and facilitated diffusion). In such processes, dissolved substances spontaneously move from a region of higher to one of lower concentration. A good example of this is the movement of glycerol from culture medium into trypanosomes [269]. Alternatively, nutrients may enter by active processes, such as active transport and endocytosis.

In active transport, substances are transported across cell membranes by carrier proteins resulting directly from the expenditure of metabolic energy. Transport mediated by carrier proteins is similar to enzyme-substrate reactions in that: 1) each carrier has a specific site for its solute; 2) when the carrier is saturated the rate of transport is maximal (saturation kinetics); and 3) solute binding is blocked by competitive inhibitors [5]. Pinocytosis by trypanosomes occurs by uptake of water and dissolved solutes (fluid-phase pinocytosis) or by receptor-mediated pinocytosis, or both [39]. Pinocytosis commonly occurs in the region of the flagellar pocket [106, 126, 166, 200, 310] and the cell coat selects the material to be ingested [69]. Similarly, uptake of macromolecules occurs by phagocytosis. Meyer and De Souza [197] demonstrated that amastigotes are able to ingest cytoplasm of the host by intracellular phagotrophy.

Energy metabolism. To better understand carbohydrate metabolism of trypanosomes, the sequence of events in the life history will be briefly presented. Physiological and ultrastructural changes associated with these events also will be considered (reviewed by [241]). *Trypanosoma brucei* subspecies will be used as the representative species, as energy metabolism for this group is better characterized than that of any other trypanosome species.

With a single bite the tsetse fly (*Glossina* spp.) may inoculate a host with thousands of metacyclic trypomastigotes. A small sore, which resolves after 1 or 2 wk, often develops at the site of inoculation. The trypanosome then gains entry into the blood and the lymphatic system. Bloodstream trypomastigotes reproduce rapidly and invade all organs of the body. In its vertebrate host, trypanosomes tend to be polymorphic, ranging from slender trypomastigotes with a long free flagellum through intermediate forms to short stumpy forms with no free flagellum. Anterior to the kinetoplast, the slender trypomastigote has a mitochondrion with sparse, short tubular cristae. Understandably, this stage is entirely dependent on glycolysis for its energy production. The mitochondrion became increasingly more elaborate as long slender trypomastigotes developed into intermediate and stumpy forms. Short stumpy trypomastigotes, the only stage infective to the tsetse possess mitochondria with many tubular cristae.

Correspondingly, these forms have a partially functional Krebs cycle but still lacked the classical cytochrome system.

After a susceptible tsetse feeds on an infected vertebrate host, trypomastigotes (located in a blood clot in the vectors midgut) multiply for ~10 days. These midgut forms have an elaborate mitochondrion with an entirely functional Krebs cycle and cytochrome chain. Eventually, the trypomastigotes migrate forward, enter the salivary gland, and differentiated into epimastigotes. After reproducing asexually, the epimastigotes transform into the metacyclic form that has a mitochondrion much like the bloodstream trypomastigotes.

Substances potentiating growth of *Trypanosoma* spp.

Blood is one of the basic requirements for growth of trypanosomes *in vitro*. Thomson [273] successfully cultivated *T. danilewskyi* by inoculating infected fish blood on agar containing ox flesh and rabbit blood. More recently, other species of trypanosomes have been routinely cultured in diphasic medium with blood agar and a liquid overlay [66, 75, 149, 151, 222, 258]. Evidently, serum related factors within blood facilitates growth, as numerous trypanosome species could be propagated in semi-defined liquid medium [35, 188, 222, 264, 277]. Clearance of plasma proteins by *T. brucei* bloodstream forms was estimated to be 0.069 μl [mg cell protein] $^{-1}$ h^{-1} [62]. Using chromatographic separation of serum, fractions that facilitated parasite growth were identified [115, 206]. Greenblatt (1969) described active factors in the serum to have an electrophoretic mobility of β-globulins.

Certainly, serum is a rich milieu of proteins, minerals, carbohydrates, fats, and vitamins that are necessary for differentiation and multiplication of blood-borne parasites. The purpose of this communication is to present a treatise of the substances that serve as growth enhancing factors for *Trypanosoma* spp. I believe that such an account affords a better understanding of the host-parasite relationship.

Influence of cells on the cultivation and differentiation of trypanosomes. *In vitro* growth of *Trypanosoma lewisi* [116], *Trypanosoma musculi* [7, 8, 10, 278], and *T. brucei* [205] was successful when the organisms were co-cultivated with spleen cells. Comparison of the ability of splenocyte populations to provide growth promoting substances revealed that the adherent, macrophage-rich population supported parasite growth much better than did the nonadherent population [7, 8, 116]. In like manner, growth of trypanosomes was enhanced in the presence of the J774.G8 murine macrophage cell line [278] or macrophages isolated from the peritoneum [8, 20, 24, 277, 278]. The role of macrophages in regulating *in vitro* growth of trypanosomes was further supported by culturing *T. musculi* with spleen cells acquired from mice inoculated with silica particles. Under these conditions, the magnitude of parasite growth was about one-tenth of that in cultures containing untreated cells [7].

At present, it is unclear if supportive activity of macrophages was associated with an immunological phenomenon. Viens *et al.* [277] reported that peritoneal cells isolated from an immune mouse support growth of *T. musculi* as well as those acquired from a normal mouse. Conversely, other studies on *T. musculi* [7] or *T. lewisi* [116] demonstrate that splenic macrophages obtained from previously infected animals do not support trypanosome growth as well as cells from normal donors.

Species specificity for *T. musculi* support was lacking, inasmuch as rat cells [8, 277, 278] and human cells [278] supported parasite growth. However, it is important to note that interstrain variation existed, as macrophages of C57BL/6 mice did not support trypanosome growth[9].

Using cell-free supernatants or double compartment vessels it was determined that growth of cultured trypanosomes was facilitated by soluble macrophage substances [7, 8, 20, 277].

Fibroblast-like cells from lung [47, 83, 130, 131, 133], spleen [31, 83], heart [47, 83], or cell lines [1, 26, 171, 195, 266, 272] were used to cultivate several species of trypanosomes. After the addition of trypanosomes to a fibroblast feeder layer, numerous parasites localized in the intercellular spaces [1, 47, 49, 130, 133, 201, 266]. In some cases, parasites were in brief contact with the surface of the feeder cell [133, 266] with no evidence of desmosomes or hemidesmosomes [49, 266], while in other reports, the parasites attached to the fibroblasts [1, 26, 83]. Whether parasites attached to the surface or not, many free trypanosomes were located in the supernatant [1, 26, 47, 49, 133, 201, 266].

Fibroblasts appear to induce trypanosome proliferation by short-range inductive signals, because conditioned medium [171, 266], lyophilized and freeze-thawed fibroblasts did not support growth [266]. Interestingly, trypanosome growth continued even after the fibroblast feeder layer degenerated, as long as medium was changed regularly [26, 272]. Thereby suggesting that fibroblasts were involved in establishing parasite cultures, but not necessarily essential for maintenance.

When mammalian trypanosomes were incubated in the presence of bone marrow cells, *in vitro* growth was greatly enhanced [21-23, 193]. The marrow feeder cell layer was so effective for parasite cultivation such that a trypanosome population was successfully established in > 97% of the wells when 10 *Trypanosoma gambiense* were seeded into a microtitre plate [23]. The ability of marrow cultures to potentiate trypanosome growth coincided with the presence of adipocyte-epithelioid aggregates [21]. Although these observations correspond to the kinetics of cellular elements involved in haemopoiesis, the growth requirements for trypanosomes were different, as marrow cells from Sl/Sl^d mice (unable to support haemopoiesis efficiently) allowed propagation of *Trypanosoma rhodesiense, T. gambiense*, and *Trypanosoma congolense* [22].

Influence of molecules on the cultivation and differentiation of trypanosomes.

Monosaccharides. Because procyclic [270] and bloodstream forms [118, 211, 269] do not actively accumulate intracellular stores of utilizable energy, exogenous sugar was an important carbon source to maintain ATP (adenosine triphosphate) production. At lower concentrations, the uptake of glucose was hyperbolic and followed Michaelis-Menten kinetics, while at higher concentrations the system was essentially saturated [118, 252, 269]. Like glucose, the uptake of fructose, mannose [252], glycerol [252, 269], and the non-metabolizable glucose analog 2-deoxyglucose displayed saturation kinetics [118, 220, 252].

Competitive inhibition assays indicated that there were two sites involved in the movement of hexoses across the cell membrane. Fructose and glucoseamine entered via the first carbohydrate transport site. The importance of carbon five in fructose transport was demonstrated by the failure of L-sorbose to inhibit fructose transport [253]. Glucose and mannose were transported through a "glucose site" [252]. The substrate binding to transport mechanisms was specific to carbon one, three, four, and six of the glucose molecules [253]. Moreover, this transport was stereospecific, as L-glucose uptake was negligible [118, 269] and did not inhibit the transport of D-glucose [220, 269].

Observations of uptake kinetics, stereospecificity, and potent inhibitors of sugar uptake have collectively given evidence that glucose was taken up by a carrier-mediated transport mechanism[118]. Reports from the literature suggested that the mode of glucose uptake was dependent on the life-cycle stage. For example, Gruenberg *et al.* [118] reported that glucose uptake by bloodstream forms of *T. brucei*, was not energy dependent (i.e., no ATP) and the uptake was driven by a downhill concentration gradient of free substrate. In contrast, Parsons and Nielson (1990) suggest that, in *T. brucei* procyclic forms, active transport (involving cotransport of a counterion down its concentration gradient) provides energy for glucose transport. The transport activity of 2-deoxyglucose, by procyclic cells was about one-sixth the rate of glucose transport of bloodstream forms. Nonetheless, glucose uptake by procyclic forms was sufficient for their survival for at least 2 hr [220].

If initial glucose concentration was maintained by replenishment, the differentiation of *T. cruzi* was blocked [53]. Exhaustion of carbohydrates in culture medium coincides with production of pyruvate [24, 49], acetate [55, 268], and organic acids [3, 6, 55, 268]. Consumption of organic acids (i.e., succinic and malic acids) by trypanosomes triggers the differentiation process [53, 97]. The need for organic acids for cell differentiation was further supported by adding exogenous acetic and pyruvic acid to glucose-free medium [97].

Amino acids. Over the last four decades, numerous studies have evaluated the use and excretion of amino acids by cultured mammalian trypanosomes. Rather than provide a extensive review of these studies, I will present key concepts from a few representative studies. A large number of studies have examined the growth requirements of trypanosomes by analyzing the amino acid composition of the cultivation medium. One of these studies [76] reported that cultivating trypanosomes for seven and 14 days resulted in a decrease (45 and 49%, respectively) in the total amino acid concentration. Collectively, several workers showed a decrease in 17 amino acids during trypanosome growth. Glutamine and threonine were consumed to the greatest extent [46, 64, 76], while alanine and glycine were the major amino acids excreted [48, 64, 76].

Other individuals have assessed growth requirements of trypanosomes by incorporation of radioactive amino acids [171] or by single component addition [77, 78] or deletion experiments [208]. The latter study illustrated that the amino acid requirements were dependent on the temperature and the age of the culture. More specifically, *T. cruzi* epimastigotes had greater requirements for amino acids at a higher temperature (18 amino acids depleted at 26°C versus seven depleted at 37°C). Also, cultures resulting from a second passage required more amino acids and amino acid precursors than the first passage. Use of amino acids may also be contingent on the availability of other nutrients, as proteases of the cysteine type (50 and 60 kDa) were more prevalent in medium depleted of glucose [218].

Purine bases, nucleotides, & nucleosides. Growth requirements and uptake of purines changes according to the life-cycle stage and to the phase of the growth cycle. When hypoxanthine, adenine, guanine, or xanthine (or their ribonucleosides) were added to purine-free medium, growth of culture forms proceeded normally [32]. Moreover, the pattern of purine uptake in *T. cruzi* epimastigotes can be predicted from the growth of cultures [120, 121]. In contrast purine uptake and purine bawee pools are insufficient to sustain growth or prolonged proliferation of amastigotes and trypomastigotes. Interestingly, when corrected for cell size, the maximal uptake capacities of amastigotes and trypomastigotes, respectively were one-fourth and five times the capacity of rapidly growing epimastigotes [121].

Iron complexes. Trypanosoma cruzi was unable to synthesize enzymes (i.e., δ–aminolevulinic acid dehydratase, porphobilinogenase, and deaminase) that were essential to the porphyrin pathway. Consequently, heme compounds were necessary as a growth factor for the flagellate [238]. Moreover, *T. cruzi* was shown to require both a heme and a chelatable iron for optimal growth [164]. Uptake of the ferritin iron complex by bloodstream forms of *T. brucei* subspecies was by endocytosis in membrane-limited vesicles present on the flagellar pocket [45, 166]. These vesicles became continuous with the collecting membrane system that connects to digestive vacuoles [166].

Addition of hemin to *T. cruzi* cultures simultaneously increased the growth rate and differentiation of epimastigotes to metacyclic forms [16]. Accordingly, replication of *T. cruzi* was reduced in a dose-dependent fashion when the iron chelator desferrioxamine was added to culture medium [187]. However, when mice were treated with exogenous iron or desferrioxamine, the intensity if the infection was not significantly altered [164]. The latter report was supported by work done by Dvorak *et al.* [79] who suggested that the intraspecific differences in the uptake of iron accounts for differences in pathogenicity.

The role of heme as a sole growth promoting factor has been questioned, as neither cytochrome C nor myoglobin were able to induce continuous growth of *T. cruzi* [19]. Certainly, additional work is necessary to determine if iron's growth promoting mechanism is associated with protein biosynthesis or iron binding capacities.

Cytokines. *Trypanosoma brucei* has two membrane polypeptides with considerable homology to the mammalian epidermal growth factor receptor [128, 129]. Presumably, binding of epidermal growth factor to this receptor evokes a mitogenic effect on *T. brucei* trypomastigotes [257] and procyclic forms by activating protein kinases and consequently altering the rate of cell division [128, 129]. This phenomenon may be important in establishing small inocula of metacyclic forms after a tsetse bite, as required concentrations (20 to 200 nM epidermal growth factor) for enhanced growth were present in tissues but not in the bloodstream [257].

A study by Ouissi *et al.* [212] demonstrated that fibronectin proteolysis by a parasite enzyme provides growth factor-like activity for the differentiation of *T. cruzi* trypomastigotes to amastigotes. These authors observed that when trypomastigotes were incubated in purified fibronectin fragments, the gelatin and heparin binding domains (40 and 45 kDa, respectively) promoted morphological transformation. Moreover, these fragments were capable of stimulating protein synthesis, increasing cyclic AMP concentration, and the phosphorylation of several parasite proteins [212].

Growth of bloodstream trypanosomes required transferrin [240]. Several studies have indicated that uptake of transferrin was a receptor-mediated process. The binding of transferrin to trypanosomes was concentration dependent and saturable [179]. Secondly, binding of transferrin was specific, as homologous proteins competed for corresponding receptors [62]. Thirdly, immunofluorescent studies, using goat anti-transferrin IgG, demonstrated that ≥ 90% of *T. cruzi* amastigotes bind human transferrin [179].

Ultrastructural studies illustrated that the receptors were associated with the flagellum and the membrane of the flagellar pocket [62, 111]. After incubation (37°C), transferrin attained an intracellular location in the parasite [179], specifically within the early endosomal compartments and in lysosome-like organelles [111]. However, transferrin does not serve directly as a growth enhancing factor since apotransferrin did not facilitate trypanosome growth [179]. Rather trypanosomes derive iron from the uptake of host transferrin [62, 179, 240].

While numerous investigators reported that tumor necrosis factor (TNF) adversely affects trypanosomes, Kongshavn and Ghadirian [160, 161] demonstrated that TNF exerts a direct anti-trypanosomal effect while simultaneously promoting *T. musculi* growth. This TNF growth promoting activity was observed when: 1) TNF was administered to mice *in vivo* from the early stage of infection onwards; or 2) parasites were incubated *in vitro* with peritoneal exudate cells and TNF. This enhancing effect of TNF was evidently mediated by macrophages or the release of monokines, as parasite growth in cultures was inhibited in the presence of only TNF [161].

Table 1-1. A general summary of classification, transmission, host range, and tissue affinity of fish protozoa[a].

Protozoan	Phylum	Transmission	Host range	Tissue affinity
Ichthyophthirius multifiliis	Ciliophora	Direct	All freshwater fish	skin or gill epithelium
Tetrahymena pyriformis	Ciliophora	Direct; facultative	Stressed fish	surface tissue central nervous system muscles
Amyloodinium spp.	Sarcomastigophora	Direct	Warm water fish	skin or gill epithelium
Cryptobia salmositica	Sarcomastigophora	Direct Indirect; leeches	Pacific coast of North America	blood body surface mucus gills
Cryptobia (T.) borelli	Sarcomastigophora	Indirect; leeches	European freshwater fish	blood
Trypanosoma burresoni	Sarcomastigophora	Indirect; leeches	Eels of eastern Canada	blood
Trypanosoma catostomi	Sarcomastigophora	Vector unknown	Suckers of North America	blood
Trypanosoma granulosum	Sarcomastigophora	Indirect; leeches	*Anguilla* spp.	blood
Trypanosoma murmanensis	Sarcomastigophora	Indirect; leeches	Northern Atlantic Ocean	blood
Trypanosoma phaleri	Sarcomastigophora	Indirect; leeches	North American freshwater fish	blood
Glugea plecoglossi	Microspora	Direct; orally	Freshwater fish	connective tissues
Glugea stephani	Microspora	Direct; orally	Northern Atlantic & European flatfish	intestine
Tetramicra brevifilum	Microspora	Direct; orally	European turbot	body wall and organs
Goussia carpelli	Apicomplexa	Direct; orally	*Cyprinus* *Carassius* spp.	intestine

[a] adapted from ([151, 186, 301])

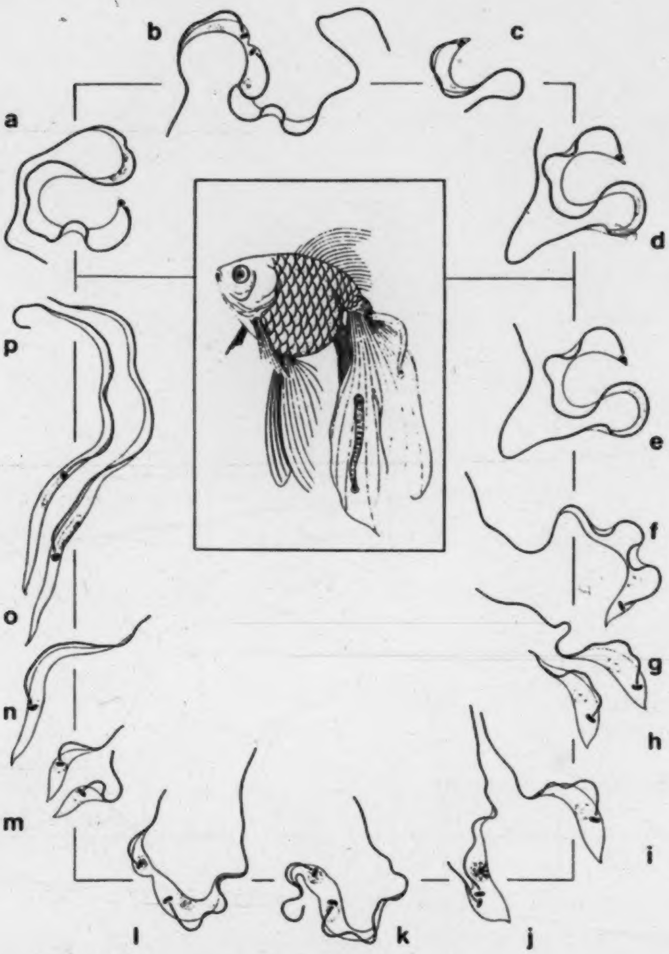

Fig. 1-1. Life cycle of *Trypanosoma danilewskyi*. Bloodstream-forms (a to d) in fish divide rapidly (b) during the early stages of infection. Soon after the leech ingests bloodstream forms (e and f), the parasite decreases in size with a concurrent shortening of the undulating membrane and free flagellum. The trypanosomes differentiate into stumpy-forms (g and h), which may undergo binary division. The parasite transforms into the crithidial form (i) that divide repeatedly (j to n). Subsequently, the parasites assume a leptomonad-and slender-form (not shown). The final stage of development results in metacyclic trypomastigotes (o and p), which serve as the infective stage. Reprinted from Developments in Aquaculture and Fisheries Sciences, Volume 26, J. Lom & I. Dykova, page 36, (1992), with permission from Elsevier Science.

CHAPTER II

GENERAL MATERIALS AND METHODS

Fish

Goldfish, common carp, or tin foil barb, purchased from Ozark Fisheries Inc. (Stoutland, MO), Grassy Forks Fisheries (Martinsville, IN) or Mount Parnell Fisheries Inc. (Mercersburg, PA) were maintained in the Aquatic Facility of the Biological Sciences Centre, University of Alberta. Fish were maintained at 20°C in tanks that received a constant flow of aerated and dechlorinated water. Nu-way grower-finisher ration pellets (Unifeed, Calgary, AB) were provided to satiation. Before manipulation (i.e., bleeding, inoculating, or marking), the fish were anesthetized by immersion in a solution of tricaine methane sulfonate or 2-phenoxyethanol. When necessary, fish were marked by fin clipping or by piercing the operculum with a metal tag (National Band and Tag Co., Newport, KY).

Parasite

Trypanosoma danilewskyi (strain TrCa) was isolated from a crucian carp by J. Lom in 1977. Our laboratory obtained this parasite from P.T.K. Woo, University of Guelph, ON. Unless stated otherwise, parasites (used for preparation of antigen, inoculation of fish, or *in vitro* cultivation) were obtained from stock cultures that were passaged 5 to 7 days beforehand.

Maintaining parasite stock cultures

Trypanosome cultures were maintained at 20°C in goldfish leukocyte medium (GFLM) (Table 2-1) [285] containing GFS [35]. Cultures were passaged every 4 or 5 days by: 1) pelleting *T. danilewskyi* by centrifugation (400 X *g*, 10 min), decantation of the supernatant, and resuspension of parasites into flasks (75 cm^2 surface area) containing 30 ml of fresh medium; or 2) subculturing parasites in fresh medium at a ratio of 1:10.

To prevent the selection of a subpopulation of *T. danilewskyi*, cultures that experienced a crisis were discarded. New cultures would be derived from experimentally infected goldfish. Fortunately, in all cases, the culture dynamics and the resulting course of infection was confirmed to be comparable to that previously observed.

Infection of fish

Before inoculation, blood was examined using the haematocrit centrifuge technique [296] to ensure that fish were free of haemoflagellates. Using a 23-guage needle and a syringe, goldfish were inoculated intraperitoneally with *T. danilewskyi* or an equal volume of control medium. With the exception of the fish used for the production of kidney leukocyte cultures that received 5 X 10^6 parasites/fish, all experimental fish were inoculated with approximately 6.25 X 10^6 trypanosomes.

Assessment of infection

To determine the number of parasites present in the peripheral blood, a small amount of blood was withdrawn from the caudal vein of each fish. After diluting the blood in tri-sodium citrate anticoagulant (100 mM tri-sodium citrate, 40 mM glucose, pH 7.3) trypanosomes were enumerated using a haemocytometer. Alternatively, heparinized capillary tubes (Fisher Scientific Co., Pittsburgh, PA) and the heamatocrit centrifuge technique [296] were used for analysis of blood obtained from fish with low parasitemia. Using the latter method, a statistical study of the sensitivity of detection of trypanosomes (*T. brucei*, *T. rhodesiense*, and *Trypanosoma evansii*) estimated that 85% of all parasites were detected [308].

Occasionally no trypanosomes were observed on the haemocytometer; however, an innumerable number was present in the heparinized capillary tube (i.e., artifact associated with the detection limit of the technique). Under these circumstances, the blood sample (diluted in the anticoagulant solution) was collected in a separate capillary tube, centrifuged, and examined.

Because enumeration of parasites by microscopy was very laborious, I tried to automate this procedure using flow cytometry. Unfortunately, this effort was met with limited success (data not presented). Based on cell size and internal complexity, the flow cytometer was unable to distinguish leukocytes from trypanosomes. Further experimentation would have been required for the establishment of a third parameter that could discern trypanosomes from leukocytes.

Purification of parasites from blood

Trypanosomes were purified from the blood according to the method of Carrington [54], with modifications. Briefly, tri-sodium citrate anticoagulant was added to the blood to prevent clotting. Parasite suspensions were centrifuged for 10 min at 750 X *g*, followed by decantation of the supernatant. With minimal disturbance of the sedimented erythrocytes, the trypanosomes were suspended in separation buffer (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 4 mM KCl, 5 mM glucose, 80 mM sucrose, pH 8.0). Centrifugation and suspension was repeated twice to remove most of the erythrocytes and serum proteins. Following the last centrifugation, the vial was tilted to a twenty-degree angle for 3 to 5 min, so that the trypanosomes would separate from the pelleted cells by settling on the wall of the vial. *Trypanosoma danilewskyi* was then aspirated and suspended in GFLM.

Preparation of fish serum/plasma

Fish serum or plasma was obtained by bleeding fish from the caudal vein using a 23 (fish >12 cm standard length) or 25 gauge (fish < 12 cm standard length) needle and a syringe. After allowing the whole blood to clot, it was centrifuged (1560 x g, 30 min) and the supernatant collected. Serum used for maintenance of cultures (i.e., trypanosomes or kidney leukocytes) was heat-inactivated (56°C, 30 min), filter-sterilized (Millipore 0.22 µm), and frozen at -20°C until needed.

For immunoassays, small quantities of plasma were obtained from immunized fish. After determining the red cell volume (described later), the glass capillary tube was etched and broken above the buffy coat. Immediately thereafter, plasma was extracted from the tube using a 1 ml syringe fitted with an adapter for capillary tubes (Bio-Rad Life Sciences Division). Samples were stored at -20°C until needed.

Isolation of peripheral blood leukocytes (PBL)

Blood, collected from infected (112 dpi) and naïve fish, was diluted four-fold in GFLM containing 40 international units (U) heparin ml^{-1} or 0.1 mM ethylenediaminetetraacetic acid (EDTA). The cell suspension was layered over an equal volume of Ficoll-Paque (Pharmacia Biotech) and centrifuged 350 X *g* for 24 min. Cells, collected from the medium/Ficoll-Paque interface, were washed two times by centrifugation in serum-free medium (450 X *g*, 10 min). Nine millilitres of sterile milli-Q-water was added to the cell suspension to lyse residual erythrocytes. After haemolysis (1 to 2 s), 1 ml of sterile 10X phosphate-buffered saline (PBS) was added to prevent leukocytes from lysing. After centrifugation, (450 X *g*), viability of cells was determined using trypan blue staining.

Kidney leukocytes

Isolation of leukocytes. Goldfish kidney macrophages were isolated as reported [203], with slight modifications. Kidneys were pressed through stainless steel mesh in medium containing 40 U ml^{-1} heparin. The cell suspension was layered over 51% Percoll and centrifuged at 400 X *g* for 25 min. Cells, collected from the medium/Percoll interface were washed three times by centrifugation in serum-free medium (300 X *g*, 10 min). After determining cell viability using trypan blue, 25 cm^2 flasks containing medium (supplemented with 100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 10% FBS, and 5% GFS) were inoculated with 3 X 10^5, 1 X 10^5, or 1 X 10^4 cells ml^{-1}. To avoid a mixed lymphocyte reaction, separate flasks were set up for the leukocytes derived from individual fish. Control flasks contained medium supplemented with antibiotics and serum but no cells.

Production of macrophage-conditioned medium. Leukocyte cultures and control flasks were incubated 18 days at 20°C. Supernatants were collected from the cultures after 6, 12, 15, and 18 days. After vigorously shaking the flask, 1 ml of the suspension was withdrawn and centrifuged (300 X *g*, 10 min). Supernatants were then collected and stored at -20°C until needed.

Cell lines

Maintenance. Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL Life Technologies, Inc, Gaithersburg, MD) supplemented with 10% FBS was used to maintain P388D.1 (American Type Culture Collection, Rockville, MD (ATCC) #CCL 46) and L929 (ATCC #CCL 1) cells that were derived from mouse lymphoid cells and connective tissue, respectively. The U937 human monocyte cell line (ATCC #CRL 1593) was maintained in RPMI 1640 (Rose Park Memorial Institute) medium (Gibco BRL) containing 10% FBS. Minimum Essential Medium with nonessential amino acids, L-glutamine, Hanks' balanced salts (Gibco BRL), and 15% FBS was used to cultivate the goldfish fibroblast-like cell line (ATCC #CCL71).

Production of cell-conditioned medium. For each cell line, six flasks (25 cm^2) were inoculated with ~10^5 viable cells ml^{-1}. Mammalian cell lines were maintained at 37°C in a 5% CO_2 environment, whereas CCL71 cultures were kept at room temperature. Control flasks contained culture medium supplemented with FBS. Supernatants were collected from the cell lines at shorter time intervals because the generation time of the cell lines differed markedly from that of the *in vitro* derived goldfish kidney macrophages.

Cultivation in cell-conditioned medium

Cell culture supernatants were used to determine the effects of cell products on the *in vitro* growth of *T. danilewskyi*. Parasites were washed by centrifugation (400 X *g*) and inoculated into 6.4 mm wells. Approximately 1 X 10^5 trypanosomes were seeded into wells containing 200 µl of cell-free supernatant or control medium. After 7 days of incubation at 20°C, trypanosomes were enumerated using flow cytometry.

Enumeration of parasites

Light microscopy. Trypanosomes were enumerated on an improved Neubauer haemocytometer, fitted with a glass coverslip (22 X 22 mm, #1 thickness), using bright field microscopy (400 X magnification).

Flow cytometry. Cultivated parasites (i.e., in the absence of any other cells) were enumerated using a FACS Calibur System (Becton Dickinson and Company, San Jose, CA) equipped with an argon laser set at 488 nm. Trypanosomes were suspended in PBS (0.26g $NaH_2PO_4 \cdot H_20$, 1.19g Na_2HPO_4, 8.77 g NaCl, and up to 1 l milli-Q-water, pH 7.2) as sheath fluid through a 70 µm flow tip. Each sample was analyzed for 30 s at a flow rate of 60 µl per min. Data were obtained using a bit-map format gated on forward light scatter versus ninety-degree light scatter.

XTT viability assay

Cell viability was assessed as previously described, with modifications [231, 243]. Each well received 50 µl of Dulbecco's PBS (Gibco BRL, Grand Island, NY), containing 1 mg/ml XTT tetrazolium salt (ICN Biochemical Inc., Aurora,) and 50 µg/ml co-enzyme Q (Sigma Chemical Co., St. Louis, MO). Plates were incubated for 3 hr at 20°C. Optical densities were determined using a microplate autoreader (Biotek Instruments Inc.) set at 450 nm.

Wright's stain technique

The Cytospin 2 centrifuge (Shandon Southern Products Ltd., Astmoor) was used to concentrate cultured trypanosomes on microscope slides (43 X *g*, 7 min). Specimens were then stained using Wright's stain (Fisher Scientific Co.). Briefly, dried specimens were fixed in 70% ethanol. The slides were then treated with: 1) eosin Y solution for 1 min; 2) methylene blue solution for 1 min; 3) and milli-Q-water to remove excess stain. Coverslips were mounted onto slides with Permount solution (Fisher Scientific Co.).

Antigen preparation

Sodium perchlorate treatment. After washing the trypanosomes in GFLM (400 X *g*, 10 min), they were killed with sodium perchlorate [156]. In brief, 10 ml of 3.33 M sodium perchlorate was added to 3 ml of the parasite suspension or control medium. After mixing for 5 min at room temperature, 10 ml of Dulbecco's PBS (Gibco BRL) was added. The parasite suspension and control medium was dialyzed (3500 molecular weight cutoff Spectra/Por Membrane, Houston, TX) extensively against PBS to remove the sodium perchlorate. The solutions were then centrifuged for 10 min at 20,000 X *g*. The pellet was suspended to the original volume of the suspension with PBS.

Preparation of trypanosome lysate. Whole cell lysate was prepared by washing (400 X *g*, 10 min) trypanosomes twice in GFLM. The parasites were then subjected to four cycles of rapid freeze-thaw. Goldfish leukocyte medium that was centrifuged, frozen, and thawed served as control medium.

Preparation of water-soluble extracts. After washing *T. danilewskyi* in phosphate saline-glucose (PSG 3:7) (400 X *g*, 10 min), soluble extracts were prepared as described [123]. Briefly, trypanosomes were killed and disrupted by three cycles of rapid freeze-thaw and sonication (Kontes Micro-Ultrasonic Cell Disrupter, Vineland, NJ). Thereafter, particulate material was removed from the suspension by centrifugation (100,000 X *g*, 1 h) and filtration (0.22 μm) of the supernatant. Control solution was prepared in a like manner using PSG 3:7. Samples were stored at –20°C until needed.

Preparation of detergent-soluble extracts. Detergent-soluble molecules were purified from *T. danilewskyi* using Triton X-114 solution [41]. Trypanosomes were washed with GFLM at 400 X *g* (twice for 10 min) and solubilized on ice in membrane solubilization buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-114, 1μg/ml leupeptin, 1 μg/ml antipain, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, 2.5 mM iodacetamide) for 1 hr. One millilitre of the parasite solution was layered onto 0.5 ml of 6% sucrose membrane solubilization buffer, preheated to 30°C. After 3 min of incubation at 30°C, the samples were centrifuged at room temperature for 10 min (300 X *g*). The oily droplet at the bottom of the tube was collected and used for immunization of fish, *in vitro* cultivation, and gel electrophoresis. Using GFLM, control solution was prepared in a similar manner.

Collection of excretory-secretory products. After two washes (400 X *g*) in GFLM, the parasites were adjusted to a density of ~4.29 X 10^8 ml^{-1}. During these preparatory steps, the parasites were kept at ~4°C. The suspension was then incubated at 20°C for 1 hr and centrifuged (400 X *g*). Remaining parasites were removed by centrifugation at 12,000 X *g* (10 min). Supernatants were carefully removed and stored at -20°C until needed. Control solution, containing no ES products, was prepared in a like manner, using GFLM.

Fish immunization procedure

Fish were administered sodium perchlorate-treated parasites, trypanosome lysate, ES products, detergent-solubilized molecules, or water-soluble molecules using a 25-gauge needle and a syringe. Trypanosome extracts equivalent to 3 X 10^7 parasites or the appropriate control solution was administered to fish intravenously (i.e., caudal vein), intramuscularly, or intraperitoneally. Likewise, extracts were administered intraperitoneally in an equal volume (70 µl) of Freund's incomplete adjuvant (FIA) or Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, MI).

Prior to these experiments, a small group of fish was inoculated via these routes to determine if this protocol was tolerable to the fish. Thereafter, fish were grossly examined each day for adverse side effects. Regardless of the administration route, all fish tolerated the inoculum well. Further, administration of parasite extract or control medium never resulted in open lesions at the site of injection.

Determination of packed red cell volume

Fish blood, collected in heparinized capillary tubes (Fisher Scientific Co.) was centrifuged for 5 min in a Micro-haematocrit centrifuge (International Equipment Co., Needham, MA). Subsequently, red cell volume in percent was determined using a Micro-capillary reader (International Equipment Co.). Briefly, the haematocrit was determined by noting the ratio of red blood cells (i.e., measured at the red cell-white cell interface) to whole blood (i.e., measured at the plasma-air interface).

Production of anti-carp immunoglobulin

Hybridoma cells (designated WCI 12) producing monoclonal antibodies, directed against a protein determinant on the heavy chain of carp Ig [162], were kept at 37°C in a 5% CO_2 environment. Hybridoma cells were maintained in RPMI 1640 medium (Gibco BRL) supplement with 10% neonatal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100μg ml^{-1} streptomycin. Cells were passaged every other day at a subcultivation ratio of 1:4. Supernatants, containing monoclonal antibodies, were collected from the cultures after 2 days. After resuspending the cells, the suspension was withdrawn and centrifuged (300 X *g*, 10 min). Supernatants were then collected and stored at -20°C until needed.

Before applying this reagent in the immunoassays, its specificity was tested using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Immunoblotting disclosed that the hybridoma supernatant recognized a single 80 kDa band, present in GFS (Fig. 2-1). This finding was consistent with that of Wilson *et al.* [293], who reported the heavy chain of goldfish IgM to be ~79.5 kDa. Further, Marchalonis (1971) reported immunological cross-reactivity between goldfish and carp Ig, although he did not determine which polypeptides contain antigenic sites shared by Igs of these two species [189].

Immunological assays

ELISA (Enzyme-linked immunosorbent assay). The ELISA was used for the detection of parasite specific antibodies in fish exposed to *T. danilewskyi* or its antigens. Once the optimal concentration of antigen and antiserum were determined by serial dilution analyses, these variables were kept constant (Table 2-2). In brief, the antigen equivalent to 10^4, 10^5, 10^6, or 2 X 10^6 trypanosomes (50 µl) was adsorbed onto the wells of a 96-well microtitre plate. Between all incubations, the wells were washed three times with milli-Q-water and blocked for 30 min with 100 µl blocking buffer (0.17 M H_3BO_3, 0.12 M NaCl, 0.05% Tween 20, 1 mM EDTA, 0.025% bovine serum albumin, 0.05% NaN_3, pH 8.5). Three incubations followed (2 hr each at 37°C): fish serum (1: 10, 1:20, 1: 40, 1:80, 1:100, or 1:200)); mouse anti-carp Ig hybridoma supernatant (diluted 1:10); and goat anti-mouse Ig alkaline phosphatase conjugate (BD PharMingen Canada, Mississauga, ON) diluted 1: 500 in PBSN (phosphate-buffered saline-sodium azide). Following the last wash, p-nitrophenyl phosphate solution (Bio-Rad Life Sciences Division) was added to each well. After 15 min, optical densities were determined using a Microplate autoreader (Bio-tek Instruments Inc., Winooskyi, VT) set at 405 nm.

Negative controls consisted of wells in which the fish serum, anti-carp IgM hybridoma supernatant, or alkaline phosphatase-labeled antibodies were omitted. For the latter two controls, serum from naïve or trypanosome-infected (50 dpi) fish served as the primary antibody. The mean value of the negative control, resulting in the highest optical density, was subtracted from the optical density value of the relevant experimental samples. Of these resulting values, the mean of two replicate plates (tested on different days) was used for data analyses.

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Parasite extracts were separated by SDS-PAGE under reducing conditions. Briefly, parasite protein was dissolved in sample buffer (59.5 mM Tris-HCl, pH 6.8, 5.7% SDS, 9.5% glycerin, 9.5% ß-mercaptoethanol, 0.02% bromophenol blue). The solution was heated at 95°C for 5 min and applied to the gel. The samples were electrophoretically separated on 1 mm 5% stacked 12% separating SDS polyacrylamide gels.

Immunoblot analyses. After SDS-PAGE, the proteins were transferred fo nitrocellulose membranes and immunoblotted according to the method of Towbin *et al.* [274], with modifications [274]. Proteins were electrophoretically transferred to membranes for 1 hr at 100 volts in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Once the optimal concentration of antigen and antiserum was determined by titration analysis, these variables were kept constant. The membranes were treated with the following reagents: (1) blocking buffer (150 mM NaCl, 25 mM Tris-HCl [pH 7.5], 1 mM NaN_3, 1% nonfat powdered milk, 0.05% Tween 20); (2) two Tris-buffered saline containing Tween 20 (20 mM Tris, 500 mM NaCl, 0.05% Tween 20 [pH 7.5]; TTBS) washes; (3) fish serum; (4) two TTBS washes; (5) mouse anti-carp Ig diluted 1:10; (6) three washes in TTBS followed by two Tris-buffered saline (20 mM Tris, 500 mM NaCl [pH 7.5]; TBS) washes; (7) alkaline phosphatase-labeled goat anti-mouse Ig diluted 1:500 (BD Pharmingen Canada); (8) p-nitroblue tetrazolium salt and 5-bromo 4-chloro-3-indolyl phosphate in aqueous dimethyl-formamide (Bio-Rad Life Sciences Division); and (9) milli-Q-water. Molecular weights were calculated from standard curves obtained from the separation of high and low molecular weight standards (Bio-Rad Life Sciences Division).

Passive transfer of immunity

Fish (14 to 18 cm total length) were intravenously immunized with 150 µl of immune serum, collected from fish 245 dpi, or nonimmune serum. Relative to the volume that would be obtained by exsanguination, this passive transfer inoculum approximates 5 to 10% of the total blood volume.

Twenty-four hours after immunization, all fish were inoculated intraperitoneally with approximately 6.25 X 10^6 trypanosomes. A fifth group of fish served as a non-immunized infected control group. Blood was collected from each fish 0, 7, 14, and 21 dpi to monitor parasitemia.

Estimation of protein concentration

The Pierce micro BCA protein assay reagent kit was used for the spectrophotometric determination of the supernatant protein concentration. In short, serial dilutions of bovine serum albumin were used to create a standard curve. Unknown samples were diluted in the same buffer as the standards. After adding the dye reagents, the samples were mixed and heated for 1 hr at 60°C. Two hundred microlitres of each sample was added to the wells of a 96-well plate (Dynatech Laboratories Inc., Chantilly, VA) and read in an automated plate reader set at 570 nm.

Statistical analyses

Statistical tests used for the analysis of experimental data are summarized in Table 2-3. For all analyses, probability values of less than 5% were considered significant.

Some data sets required a logarithmic transformation to stabilize the variances prior to statistical analyses. In these cases, one whole number was added to each value before the logarithmic transformation.

So that results obtained from fish that died during the immunization experiments (see Chapter V) were not excluded from the analyses, the repeated measures ANOVA (analysis of variance) was calculated for data collected from days 0 to 7 separately from that collected on days 14 to 56.

When calculating the correlation coefficient of the humoral response and the parasite abundance, the ELISA values were transformed into the percent increase or decrease of that observed 0 dpi. If serum collected on 0 dpi was lost (i.e., breakage of capillary tube in haematocrit centrifuge), all data from this fish was omitted from the correlation analyses.

On each experimental day (4, 7, 14, 28, or 56) the percent of control value was defined as:

$$\%control = \frac{\frac{1}{n}\left(x_{i1} + x_{i2} + x_{i3} + \ldots\right)}{\frac{1}{n}\left(x_{c1} + x_{c2} + x_{c3} + \ldots\right)}$$

n = number of fish per group

x_i = percent increase or decrease of that observed on 0 dpi for immunized individuals

x_c = percent increase or decrease of that observed on 0 dpi for control individuals

Table 2-1. Composition of Goldfish Leukocyte Medium.[a]

Reagents or Solutions	Amount	Special Requirements
Milli-Q-water	600 ml	Milli-Q® PF Plus System[b]
Hank's solution[c]	80 ml	10X, no Ca^{2+} and Mg^{2+}
MEM amino acid solution[c]	25 ml	50X
MEM nonessential amino acid solution[c]	25 ml	100X
$NaHCO_3$	2.52 g	
NaOH	0.3 ml	1 N
Sodium pyruvate solution[c]	25 ml	10 mM
MEM vitamin solution[c]	20 ml	100X
Nucleic acid precursor solution	20 ml	2.5 mM[d]
L-glutamine solution	20 ml	200 mM
Gentamicin solution	2 ml	50 mg/ml
2-mercaptoethanol solution	2 ml	50 mM
HEPES	4 g	
Insulin	0.01 g	
GFL-15 Medium[e]	1000 ml	

[a] developed by Wang and Belosevic (1994)
[b] Purification pack (#CPMQ004R1) and filter unit (#MPGL04SK2)
[c] purchased from Gibco BRL Life Science Technologies, Inc.
[d] 2.5 mM each of adenosine, cytidine, hypoxanthine, thymidine, and uridine
[e] Leibovitz-15 and Dulbecco's Minimum Essential Medium 50:50 (v/v)

Table 2-2. Optimal concentration of antigen and primary antibody used for the ELISA.

Parasite extract	Antigen equivalence (parasites well^{-1})	Primary antibody dilution
Sodium perchlorate-treated	1 X 10^6	1 : 20
Trypanosome lysate	1 X 10^6	1 : 20
Water-soluble molecules	1 X 10^6	1 : 10
Excretory-secretory products	2 X 10^6	1 : 10

Table 2-3. Statistical analyses performed on experimental data.

Experiment	Statistical analysis								
	a	b	c	d	e	f	g	h	i
Infection dynamics (Challenge)					✓			✓	
In vitro cultivation experiments:									
Comparative assessment of sera					✓			✓	
Morphometric study	✓	✓						✓	
Cell-conditioned medium:									
Primary cultures (Transwell chambers)		✓						✓	
Primary cultures					✓		✓	✓	
Cell-lines		✓					✓	✓	
Immunization with parasite extracts:									
Parasitemia					✓		✓	✓	
Red cell volume					✓		✓	✓	
ELISA				✓		✓	✓		
Passive transfer					✓			✓	
Antigen-induced proliferation			✓					✓	
Macrophage-trypanosome interactions		✓							✓

a one-factor ANOVA
b two-factor ANOVA
c three-factor ANOVA
d two-sample t test
e repeated measures one-factor ANOVA
f Welch's approximate t-test
g correlation coefficient (r)
h least square means
i Fisher's protected least significant difference test

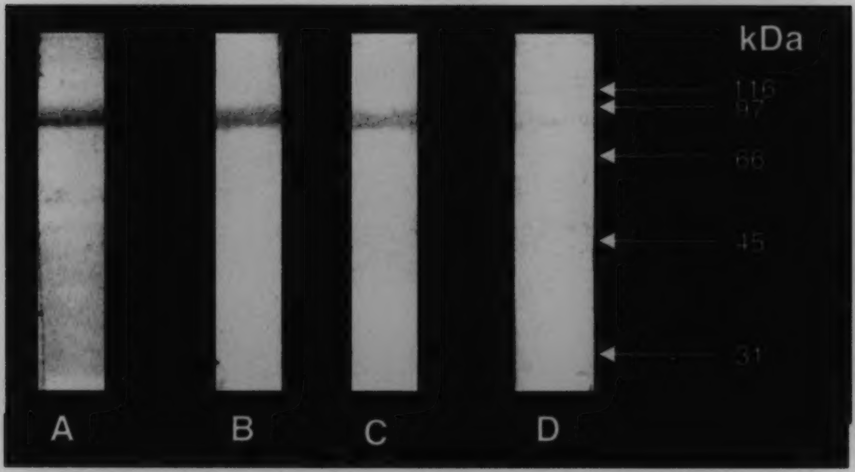
kDa
116
97
66
45
31
A
B
C
D

Fig. 2-1. Immunoblot of goldfish serum probed with neat, 1:5, 1: 10, and 1:50, anti-carp IgM hybridoma supernatant (lane a, b, c,and d, respectively). Each lane contained 0.75 µl serum.

CHAPTER III

CHARACTERIZATION OF THE COURSE OF INFECTION

INTRODUCTION

In nature the transmission of *T. danilewskyi* is by leeches (i.e., *Hemiclepsis marginata* or *Piscicola geometra*) containing metacyclic trypanosomes [27, 303]. Trypanosomes present in the crop of the invertebrate host migrate to the proboscis sheath and are transmitted when the leech feeds on the vertebrate host [225, 230]. Following inoculation of the infective stage, the parasite multiplies in the blood of fish [299]. Inasmuch as prevalence of infection and infection dynamics were affected by the physiological status of the host and environmental factors [141, 309], fish may carry an infection for a prolonged time, even years [186]. After recovering from the primary infection, fish tend to be resistant to reinfection. Protection from subsequent challenge in surviving fish has been reported to be at least 89 [140], 190 [298], and 365 [283] days after the primary infection. The main objective of studies reported in this chapter was to determine the course of *T. danilewskyi* infection (first and second exposure) in goldfish housed in the stated conditions.

EXPERIMENTAL DESIGN

Abundance of parasites in goldfish (10 to 13 cm total length), inoculated intraperitoneally with approximately 6.25×10^6 trypanosomes, was determined every 15 days. One hundred fifty dpi, fish were randomly divided into two groups. One group was rechallenged by intraperitoneal inoculation of approximately 6.25×10^6 trypanosomes per fish, while the second group was inoculated with an equal volume of control medium. To ensure infectivity of the inoculum, naïve fish were injected with *T. danilewskyi*. Parasitemia in challenged fish was determined at weekly intervals.

RESULTS

As shown in Fig. 3-1A, 82% of the fish survived the primary infection. Survival of fish was related to the intensity of infection. For example, mortality was observed in all fish with parasitemia exceeding 1 X 10^9 ml^{-1} blood. Mortality of 20% and 12% was observed in those fish where parasitemia reached 10^8 and 10^7 trypanosomes ml^{-1} blood, respectively. All fish with a maximum parasite intensity of 10^6 trypanosomes ml^{-1} blood survived the infection.

At the onset and peak of infection, two populations of fish were evident. Namely, a group of fish (23%) that never developed a patent infection and the remaining fish, which developed infections that exceeded 1 X 10^6 trypanosomes ml^{-1} blood (Fig. 3-1B and Table 3-1). In the latter group, there was a rapid onset of parasitemia during the first 15 days of infection (Fig. 3-1C). As expected with an outbred animal population, significant variability in the numbers of parasites was observed. Although peak parasitemia (median = 3.65 X 10^7, mean = 8.62 X 10^7, one standard error = 3.47 X 10^7 parasites ml^{-1} blood) occured on day 30, a similar number of trypanosomes was present in fish that were monitored 45 and 60 dpi. In many fish, a rapid decline in parasitemia occurred between 60 and 75 dpi. Notwithstanding, clearance of parasites was observed as early or late as 45 and 105 dpi, respectively (Fig. 3-1B). After the initial decline, parasites were detected intermittently in low numbers (<1000 parasites ml^{-1} blood).

Goldfish acquired resistance to *T. danilewskyi* after initial exposure and alleviation of the acute primary infection. Mortality (8%) was only observed in the group of naïve fish given a primary infection (Fig. 3-2A). The secondary challenge with *T. danilewskyi* resulted in a prevalence of infection that was similar to recovered fish that were inoculated with control medium (Fig. 3-2B). Examination of individual fish in these two groups disclosed that very low parasitemia (< 200 parasites ml^{-1} blood) was detected in one of five rechallenged fish and one of four recovered-unchallenged (control) fish. Conversely, 92% of the naïve fish developed a patent infection. The variability observed in these treatment groups was primarily due to one or two fish with parasitemias that remained around the detection limit.

Resistance to the secondary infection was independent of the intensity of the initial infection. The mean parasitemia of rechallenged fish was markedly less ($P < 0.002$) when compared to fish given a primary infection (Fig. 3-2C). However, no significant differences were observed when parasitemia in rechallenged fish was compared to the second group of recovered fish that was only inoculated with control medium.

DISCUSSION

Several workers have determined that the number of surviving fish was related to the number of *T. danilewskyi* in the inoculum [140, 183, 283, 298]. For instance, a high number of goldfish (up to 80%) died from parasitemia when inoculated with a large number of trypanosomes. This mortality was reduced considerably when goldfish were infected with a lower number of trypanosomes. The susceptibility of fish was also determined by the physiological condition of the fish [183] and environmental factors [141]. Additionally, the source of *T. danilewskyi* may influence the outcome of the disease. Sixty percent mortality was observed in fish that were inoculated with 3.8×10^5 trypanosomes, maintained in goldfish by blood inoculation [298]. In comparison, inoculation of $>1 \times 10^6$ trypanosomes, maintained using *in vitro* cultivation, was necessary to cause a similar magnitude of mortality [283]. In my experiments, inoculation of approximately 6.25×10^6 *T. danilewskyi* resulted in 18% mortality. Although these observations may suggest a loss of virulence in cultured parasites, it was possible that this was influenced by the morphological-forms present in the inoculum (see Chapter IV). This hypothesis was supported by the observation that the course of infection in fish, inoculated with freshly established cultures, was not statistically different from that observed in fish that were inoculated with parasites cultured for ~1.5 yr (see Chapter V).

The extreme variability in susceptibility of individual fish to haemoflagellates has been attributed to the general condition of the fish [184] and genetic variation among outbred fish [43, 291]. Moreover, because goldfish were not commercially available as specific pathogen-free animals, it was possible that epitopes from common parasites of fish were cross-reactive with *T. danilewskyi* antigens. As presented in Chapter V, this was unlikely, as ELISA values (0 dpi) of the presumably resistant fish were similar to that of the susceptible fish.

The course of infection observed in this study was similar to that reported previously [215, 284]. Relative to the immunological association between the host and parasite, several inferences can be drawn from the course of infection. For example, the low number of parasites detected in the blood of recovered fish suggested that resistance to a secondary *T. danilewskyi* infection was maintained by non-sterile immunity (i.e., resistance to reinfection even though the original infection persists) [140]. It was likely that low numbers of trypanosomes sequester in internal organs (i.e., kidney) and were emitted intermittently into the blood to stimulate the immune system (see Chapter IV). Secondly, the course of infection inferred that the surface coat of *T. danilewskyi* did not vary antigenically, inasmuch as multiple acute infections were not observed. This hypothesis was supported by cross-protection experiments between cultured trypanosomes (Clone K1) and lines isolated from recovered fish [215].

My observations provide corroborative evidence that goldfish acquire long lasting resistance to *T. danilewskyi* after initial exposure [140, 298]. This secondary response appeared to require minimal antigenic stimulation, inasmuch as protection against the challenge inoculum was independent of the intensity of the primary infection. Similar to that observed in unchallenged recovered fish, very low numbers of trypanosomes were detected intermittently in blood of challenged fish. Conversely, Woo (1981) did not detect parasites in fish challenged 190 days after the initial infection. It appears that this small discrepancy can be accounted for by the detection limits of microscopic examination, as other workers, using xenodiagnosis, demonstrated that fish may harbor *T. danilewskyi* 1 yr after infection [215].

Taken together, my results suggest that resistance was associated with memory cells and a secondary immune response. As discussed later (see Chapter VI), both cellular and humoral responses are believed to be essential in arresting the development of *T. danilewskyi*.

Table 3-1. Parasite intensity in naïve goldfish (n=22) that inoculated with *Trypanosoma danilewskyi*.

Parasites ml^{-1} blood ($\log_{10}$)	TIME (days postinfection) 15	30
0	6[a]	5
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	6	2
7	10	11
8	0	4

[a] number of fish possessing the indicated numbers of parasites

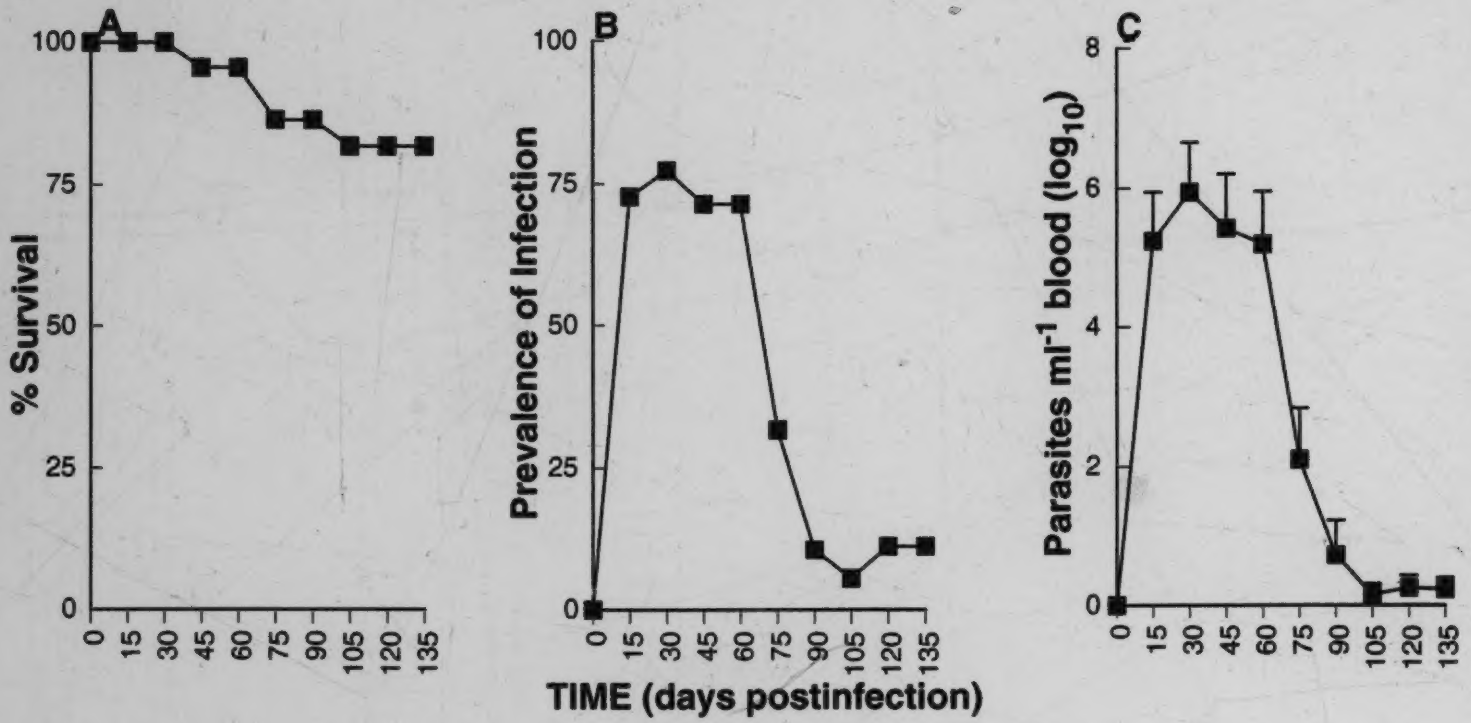

Fig. 3-1. Mortality (A), prevalence of infection (B), and course of infection (C) in naïve goldfish (n=22) that were inoculated with *Trypanosoma danilewskyi*. Data points in 3-1C represent the mean number of parasites ml^{-1} blood ± SEM.

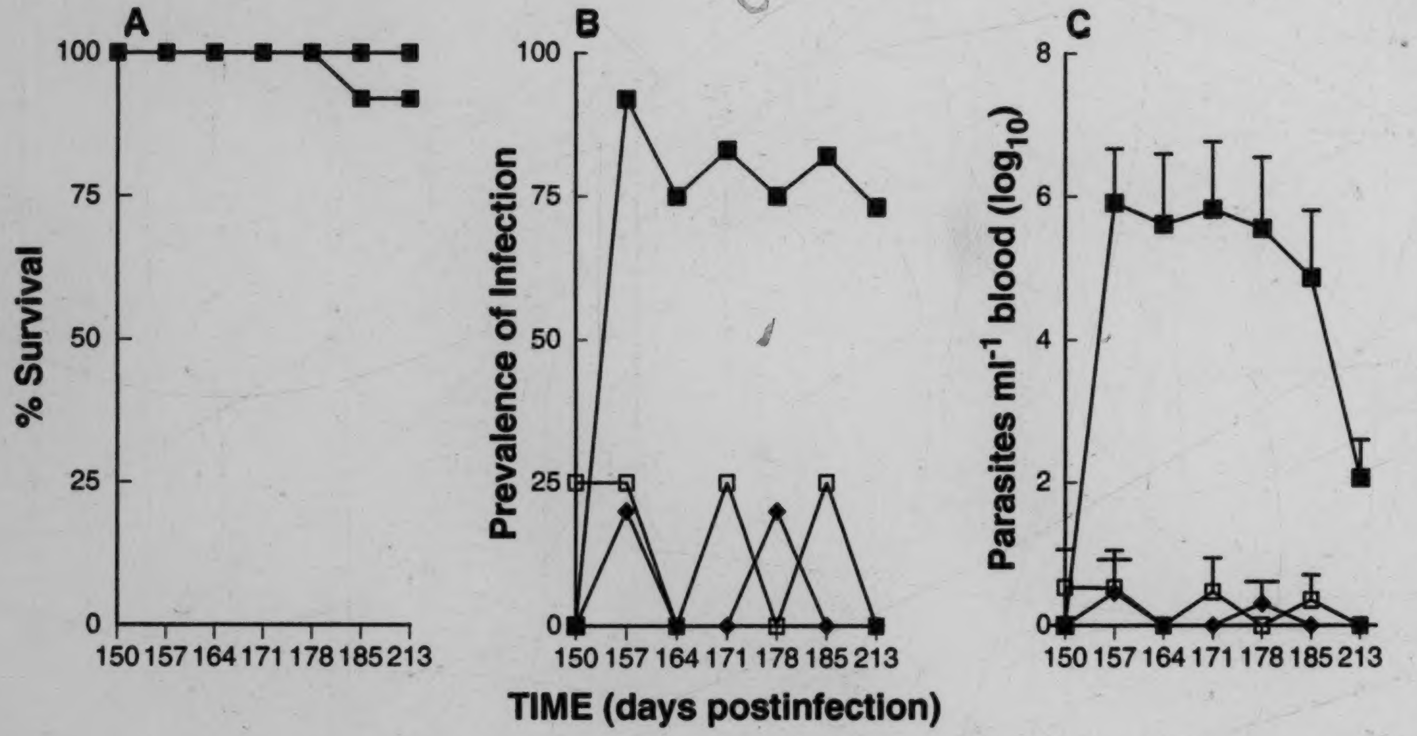

Fig. 3-2. Mortality (A), prevalence of infection (B), and course of infection (C), in ■ naïve (n=12) or ◆ rechallenged (n=5) goldfish. □, fish (n=4) that recovered from the primary infection but were not rechallenged with *Trypanosoma danilewskyi*. Data points in 3-2C represent the mean number of parasites ml^{-1} blood ± SEM.

CHAPTER IV

CULTIVATION OF *TRYPANOSOMA DANILEWSKYI IN VITRO*[*]

INTRODUCTION

Although there are 190 species of fish trypanosomes [186], little is known about the factors that stimulate division and morphogenesis of these parasites. Thomson [273] successfully cultivated *T. danilewskyi* by inoculating infected fish blood on agar containing ox flesh and rabbit blood. More recently, other species of fish trypanosomes have been routinely cultured in diphasic medium with blood agar and a fluid overlay [66, 149, 151]. Evidently, serum-related factors within the blood facilitated parasite growth, as *T. danilewskyi* [35, 141, 233, 284], *Trypanosoma granulosum* [67], and *Trypanosoma catostomi* [149] could be propagated in semi-defined liquid medium.

Most attempts to cultivate *T. danilewskyi in vitro* have been in media containing mammalian components. The suitability of mammalian sera for the cultivation of *T. danilewskyi* remains uncertain. For example, Islam and Woo [141], showed that *T. danilewskyi* did not multiply in: Hank's solution supplemented with 10% FBS; diphasic blood agar media (SNB9) with human or sheep blood; or SNB9 with sheep blood and various vitamins. In contrast, medium (consisting of basal broth, rabbit serum, and erythrocyte lysate from rabbit blood) facilitated exponential growth and indefinite subculturing [233].

While the factors that elicit fish trypanosome growth remain unidentified, Smolikova *et al.* [251] reported that proliferation may be the result of a vital reaction of the parasite to a compound of fish, as medium without the presence of living cells (presumably epithelial-like and fibroblast-like) failed to support growth. Smolikova *et al.* [251] and a subsequent study [67] demonstrated that *in vitro* growth of parasites was enhanced by exogenous insulin and hemin, respectively. Clearly, these compounds cannot be the only growth factors present in serum, as a modest increase in growth was only obtained with insulin [67]. Likewise, tissue culture medium without fathead minnow or brown bullhead cells, even with hemin supplement, failed to support parasite growth [251].

---

[*] **Portions of this chapter have been published:**
Bienek & Belosevic. 1997. Comparative assessment of growth of *Trypanosoma danilewskyi* (Laveran & Mesnil) in medium containing fish or mammalian serum. Journal of Fish Diseases 20:217-221.

Bienek & Belosevic. 1998. Macrophage or fibroblast-conditioned medium potentiates growth of *Trypanosoma danilewskyi* Laveran & Mesnil, 1904. Journal of Fish Diseases 22: 1-9

The aim of experiments described in this chapter was to better define the conditions necessary for trypanosome growth. The first objective determined whether *T. danilewskyi* can be cultivated in medium supplemented with goldfish, carp, or tin foil barb serum, and to compare the *in vitro* growth curves in serum-free medium or medium containing horse serum or FBS. Further, a morphometric study of *T. danilewskyi* was done to determine the proportion of life cycle forms present in stock cultures, maintained in 10% GFS. Lastly, I studied the association between host cell (macrophage and fibroblast) products and the growth of parasites. More specifically, the latter objective determined whether the: 1) products of macrophages, isolated from infected or uninfected fish, affect parasite growth; 2) activity of cell-conditioned medium was specific to species or cell-type; and 3) *in vitro* growth of trypanosomes correlates with the protein concentration of the cell culture supernatants.

EXPERIMENTAL DESIGN

Comparative assessment of sera

Immediately after purification of *T. danilewskyi* from the blood, they were used for initiation of *in vitro* cultures. Serum-free culture media, GFLM or Insect Xpress (BioWhittaker, Walkersville, MD), were used for cultivation. Goldfish leukocyte medium was used alone or supplemented with 10% horse serum, 10% FBS, 5% FBS and 5% GFS serum, or 10% fish serum (GFS, tin foil barb, or carp).

Flasks (75 cm^2 surface area) containing serum-free medium, mammalian serum, or GFS were inoculated with 6.7 X 10^4 parasites ml^{-1}. For these cultures, the number of trypanosomes was determined at 7 day intervals for 6 wk. Parasites were subcultured when they exceeded 6.0 X 10^5 ml^{-1}. Because of the limited amount of carp and tin foil barb serum, parasites were seeded into smaller flasks (25 cm^2 surface area) at a final concentration of 2.0 X 10^5 ml^1. For comparison, GFS was used under similar conditions. Parasites were enumerated in these cultures five and 10 days after the initiation of cultures.

At the specified intervals, the medium was changed and the number of trypanosomes per flask determined. Parasites were pelleted by centrifugation, resuspended in a small amount of medium, and enumerated with the aid of a haemocytometer. Results from two independent experiments were combined and used for data analyses of long-term cultivation.

The effect of immune serum on the *in vitro* growth of *T. danilewskyi* was determined using samples collected from infected fish (75 dpi). Serum (10%) was aliquoted into the culture wells (96-well plate) containing GFLM. Wells containing serum from naïve fish served as the control group. Approximately 1 X 10^5 trypanosomes was seeded into each well. Observations were made from independent cultures (i.e., not repeated measures) after 1, 4, and 7 days of incubation at 20°C. Parasites were enumerated using a haemocytometer.

Using serum from a different group of fish, a similar experiment was conducted to determine if samples collected at the onset or peak parasitemia (15 and 45 dpi, respectively) affected *in vitro* growth differently than sera from fish that survived the primary infection (75 dpi). Briefly, trypanosomes were seeded into wells containing modified GFLM [203] and 10% immune or nonimmune serum. Using a haemocytometer, parasites were enumerated after 9 days of cultivation.

Morphometric study of cultured trypanosomes

Life-cycle forms and total number of trypanosomes present in stock cultures were determined after 3, 6, and 10 days of cultivation. Flasks (25 cm^2) containing GFLM and 10% GFS were incubated with 2 X 10^5 parasites ml^{-1}. On the appropriate culture day, microscope slides were prepared from six independent flasks. Using bright field microscopy (1000 X) and a calibrated ocular micrometer, measurements (i.e., length, width, and distance of kinetoplastid from nucleus) were taken to distinguish the different morphological forms. On each slide, representatives of each approximate quadrant were measured.

Comparative assessment of cell-conditioned medium

As described later (see Appendix C), low numbers of trypanosomes appeared spontaneously in leukocyte cultures derived from recovered fish. Transwell microporous (0.4 µm pore size) cell culture inserts (Corning Costar Corporation, Cambridge, MA) were used in a pilot study to determine whether: 1) host cells potentiate growth of *T. danilewskyi*; and 2) growth-enhancing activity was contingent on contact between the host cell and the parasite. Kidney leukocytes were isolated and incubated at 20°C for four or 18 days. Macrophages (1 X 10^5 or 1 X 10^6) were seeded into the lower chamber containing modified GFLM supplemented with 10% FBS and 5% GFS. Control chambers contained culture medium supplemented with sera. All cell culture inserts were then seeded with 1 X 10^5 trypanosomes. After 7 days of incubation, the parasites were enumerated using a haemocytometer.

After determining the effect of host cell products on the growth of *T. danilewskyi*, an experiment was conducted to establish if the growth of *T. danilewskyi* in cell-free supernatants was proportional to the number of macrophages seeded in cultures. Briefly, leukocytes were isolated from naïve fish, seeded into flasks at a density of 3 X 10^5, 1 X 10^5, or 1 X 10^4 cells ml^{-1} medium and incubated at 20°C. Supernatants were collected from these cultures after 6, 12, 15, and 18 days. Trypanosomes were seeded into wells containing conditioned or control medium, incubated for 7 days, and enumerated using flow cytometry.

To determine if growth-enhancing activity was associated with the course of infection, supernatants were collected on day 6, 12, 15, and 18 from macrophage cultures that were derived from infected (4 to 16 wk) or uninfected fish. As described in the previous paragraph, *T. danilewskyi* was seeded into culture wells containing supernatant and enumerated after 1 wk of cultivation. A duplicate experiment was performed on a different group of fish.

Supernatant collected from macrophage/monocyte-like cells (P388.D1 and U937) or fibroblasts (CCL71 and L929) were tested to determine if growth enhancing activity was specific to species or cell-type. Supernatants were collected from the flasks at different time intervals because the generation time of the cell-lines differed. The resulting cell-conditioned medium or control medium was used for *in vitro* cultivation of *T. danilewskyi*. After 7 days of incubation, trypanosomes were enumerated using flow cytometry.

RESULTS

Comparative assessment of sera

Goldfish leukocyte medium supplemented with heterologous fish serum supported the proliferation of *T. danilewskyi*. After 10 days, the number of trypanosomes in flasks containing tin foil barb serum increased nearly seven-fold ($P < 0.0001$) (Fig. 4-1). The mean generation time in 10% tin foil barb serum was calculated to be 118.1 ± 11.9 hr. When the number of parasites, cultured in tin foil barb serum, was compared with those in GFS or carp serum, it was found to be significantly higher ($P < 0.02$). After 5 and 10 days of cultivation, flasks containing carp serum had approximately a two and four-fold increase in the number of trypanosomes, respectively. The number of trypanosomes grown in carp serum was not statistically different from those grown in GFS. Trypanosomes cultivated in 10% carp serum or 10% GFS had a mean generation time of 120.3 ± 23.56 and 134.0 ± 22.3 hr, respectively. In comparison, the generation time *in vivo* was calculated to be 300 ± 185 hr in goldfish (15 to 30 dpi) that were inoculated with approximately 6.25 $X10^6$ trypanosomes (data not shown).

For a prolonged period, *T. danilewskyi* was cultivated in medium supplemented with GFS or FBS, or both (Fig. 4-2). After the first week, the mean number of trypanosomes ml^{-1} medium was similar in all treatment groups. When compared to the initial inoculum, 2 wk of cultivation in 10% GFS resulted in a three-fold increase in the mean number of parasites. Between 2 and 6 wk, the number of parasites in these cultures increased linearly. By 6 wk, there was a 15-fold increase of *T. danilewskyi* in cultures containing 10% GFS. Although cultures containing 5% FBS and 5% GFS supported trypanosome growth over a prolonged period, the increase in parasite number was significantly less ($P < 0.0001$) than in cultures containing 10% GFS. After a lag period of 5 wk, the cultures treated with 5% FBS and 5% GFS had over a six-fold increase of *T. danilewskyi*. Conversely, in the flasks containing 10% FBS, relatively few trypanosomes (~650 parasites ml^{-1}) were alive at the end of the observation period.

Trypanosomes seeded in flasks containing no serum or 10% horse serum decreased in number over time (Table 4-1). By wk 6 and 15, few parasites (~$2 X 10^4 ml^{-1}$ and 15 ml^{-1}, respectively) were alive in those flasks containing 10% horse serum. Parasites seeded in flasks containing only GFLM medium decreased in number until 5 wk, at this time no motile parasites were observed. Similarly, Insect Xpress medium failed to support the growth of *T. danilewskyi* (data not shown).

Sera, collected from infected fish (75 dpi) supported *in vitro* cultivation of *T. danilewskyi* as well as sera collected from naïve fish (Fig. 4-3). Consistent with that reported in the previous experiment, the number of trypanosomes present after 7 days of cultivation did not differ significantly from that in the initial inoculum. Notwithstanding, maintenance of parasites was dependent on the presence of serum, as trypanosomes seeded in wells containing serum-free medium decreased ($P < 0.01$) in number over time.

The ability of serum to maintain trypanosomes *in vitro* was independent of the course of infection, as immune sera (collected from infected fish 15, 45, or 75 dpi) was able to sustain the parasites as well as control sera (Fig. 4-4). Further analysis revealed that sera from fish with light or moderate parasitemia (250 to 6.25×10^6 parasites ml^{-1} blood) supported *in vitro* growth as well as sera collected from fish with massive parasitemia ($>10^8$ parasites ml^{-1} blood) (data not shown).

Morphometric study of cultured trypanosomes

The cultures used to determine the proportion of the morphological forms were monitored over a period equivalent to approximately one passage. When compared to the initial inoculum, there was a significant increase ($P < 0.04$) in the number of trypanosomes after 3 days of cultivation (Fig. 4-5A). Between 3 and 10 days, the number of trypanosomes in these cultures increased linearly ($P < 0.0001$), with a final mean of 1.3×10^7 parasites ml^{-1} medium.

A pleomorphic population was observed on each experimental day. Notwithstanding, the predominant life-cycle stage was the bloodstream-forms (Fig. 4-5B to Fig. 4-7). A maximum of 82% of bloodstream-forms was observed at the beginning of the experiment. Thereafter, the number decreased ($P < 0.001$) and composed approximately 60% of the population. The differences between the latter observations (day 3, 6, and 10) were insignificant. Although asexual reproduction of *T. danilewskyi* has been reported in the vertebrate host [299, 303] and the leech [225], all trypanosomes with cytoplasmic and nuclear division were collectively referred to as dividing-forms. The largest number of dividing-forms was observed on the third day of cultivation, however this was not significantly different from that observed on day 0, 6, or 10.

With the exception of metacyclic trypomastigotes, morphological forms that would be observed in the leech (i.e., stumpy-, crithidial-, leptomonad-forms, etc.) were present in low numbers (Fig. 4-5B). The total number of these forms comprised between 5 to 17% of the population. Compared to that observed in the initial inoculum, the percentage of metacyclic trypomastigotes increased two- to three-fold ($P < 0.03$) by day 6 and 10, respectively.

Comparative assessment of cell-conditioned medium

Fish kidney leukocytes. Although attachment between host cell and parasite has been occasionally observed in cultures derived from recovered fish, cell to cell contact was not required for growth enhancement of *T. danilewskyi*. When compared to control wells, Transwell chambers containing 10^5 or 10^6 host cells had approximately a two-fold increase ($P < 0.01$) in the number of parasites (Table 4-2). Although a greater number of trypanosomes were observed in chambers containing 10^6 host cells than in those with 10^5, this was only significant ($P < 0.01$) in cultures that were derived 18 days before beginning the experiment.

Growth-enhancing activity of fish macrophage supernatants was relative to the number of cells per culture (Fig. 4-8). Supernatants collected on day 12, 15, and 18, from cultures containing 3 X 10^5 macrophages ml^{-1} had $\geq$ 40% increase ($P \leq 0.0001$) in the number of trypanosomes compared to cultures with 10^4 macrophages ml^{-1} or control medium. Similarly, supernatants collected from cultures containing 3 X 10^5 macrophages ml^{-1} (cultivated for 12 or 15 days) enhanced the growth of *T. danilewskyi* significantly better ($P < 0.0001$) than those collected from cultures with 10^5 cells ml^{-1}. Regardless of supernatant age, trypanosome growth in medium conditioned by 10^4 cells ml^{-1} did not differ significantly from that in control medium. Although 3 X 10^5 macrophages ml^{-1} enhanced parasite growth better than 1 X 10^5 ml^{-1}, further studies involved 1 X 10^5, macrophages ml^{-1} to accommodate the variable cell yield from fish kidneys.

Supernatants, collected from macrophage cultures derived from either infected or uninfected fish, enhanced growth of the parasites significantly ($P < 0.02$) compared to control medium (Fig. 4-9). Growth-enhancing activity of macrophage-conditioned medium was independent of the course of infection, as similar trends were observed when cultures were prepared 28, 56, 112, and 152 dpi. With the exception of 112 dpi, trypanosome growth in supernatants, collected from macrophage cultures derived from infected fish, did not differ markedly from those isolated from uninfected fish. At present, it is unclear why parasite growth in medium, conditioned by macrophages derived from fish infected for 112 days, was significantly less ($P < 0.02$) than that in medium conditioned by macrophages isolated from uninfected fish. It was unlikely that these results were associated with the dramatic decline in parasitemia observed between day 60 and 90, because no significant differences between infected (70 dpi) and uninfected groups were observed in a replicate experiment (Fig. 4-10).

Enhancement of trypanosome growth was relative to the age of the cell-conditioned medium (Fig. 4-9). Supernatants collected from cell cultures on day 15 and 18 supported growth of *T. danilewskyi* significantly ($P < 0.04$) better than those collected on the sixth day of cultivation. Compared to control medium, the average increase in the number of parasites exceeded 25% in the wells inoculated with conditioned medium that was collected 6 days after cultivation. Maximum parasite growth (~65% increase) was obtained in conditioned medium collected on the fifteenth day of cultivation.

Regardless of age, the total protein concentrations of samples collected from fish macrophage cultures were similar (data not shown). No significant differences were observed (mean protein concentrations) in samples collected from cultures established from fish infected for 28 or 112 days. Conversely, cell-conditioned medium collected from cultures, derived 56 or 152 dpi, had approximately 10% less protein ($P < 0.0001$) than the control medium. Further analysis of these samples determined a significant ($P <$

0.03) negative correlation between the protein content and the ability of macrophage-conditioned supernatants to support trypanosome growth.

Mammalian Macrophages. When trypanosome growth in undiluted supernatants from U937 cultures was compared to that in control medium, a modest but significant ($P <$ 0.0005) increase was only observed in those wells containing medium conditioned by monocytes for 1 day (Fig. 4-11). Conditioned medium, collected on day 3 and 5, that was diluted with 50% fresh medium facilitated parasite growth (~ 50 and 70%, respectively) better than undiluted conditioned medium. Furthermore, parasite numbers increased by more than 15, 35, and 60% (compared to growth in control or undiluted medium) when cultivated in conditioned medium collected on days 1, 3, and 5, respectively. With a three-fold increase over the initial inoculum, all control samples (diluted or undiluted) supported trypanosomes equally. When compared to the control group, a significant decrease ($P < 0.001$) in the protein concentration was observed in medium conditioned by U937 cells for 1, 3, or 5 days (Fig. 4-12).

Compared to the initial inoculum, a significant increase ($P < 0.0001$) in the number of parasites was observed in wells containing supernatants conditioned by P388D.1 cells or control medium (Fig. 4-13). However, wells containing conditioned medium, collected after the first day of culture, had ~40% ($P < 0.0001$) more parasites than those containing control medium, although to a lesser extent (~20% increase), medium conditioned by mouse macrophages for 3 days also enhanced trypanosome growth. In contrast, supernatants conditioned by P388D.1 cells for 5 days did not facilitate growth of *T. danilewskyi* as well. Supernatants collected from P338D.1 had significantly less ($P <$ 0.0001) protein than control medium (Table 4-3). This difference in protein concentration was observed after 3 and 5 days of culture. A ~65% decrease ($P < 0.0001$) was observed when the protein concentration of supernatants was compared over time (day 1 vs 5). In contrast, the protein concentration of control medium did not differ significantly over time. Despite the significant decline in the protein concentration in the macrophage supernatants, no strong linear correlation could be drawn between parasite growth and the total protein content. With regard to growth enhancing activity and total protein concentration, similar trends were observed in a replicate experiment that tested supernatants collected from P388D.1 cultures after 3, 6, and 9 days (data not shown).

Fibroblasts. When compared to the control group, supernatants collected from L929 murine fibroblast cultures effected a significant increase ($P < 0.0001$) in the number of trypanosomes (Table 4-4). As previously observed, dilution of the conditioned medium markedly affected the growth-enhancing activity. For example, when compared to undiluted samples, dilution of conditioned medium (collected on day 3 and 5), resulted in over a four-fold increase ($P < 0.0001$) in the number of parasites. Conversely, dilution did not markedly affect the ability of control medium to sustain trypanosome cultures. Comparable to that observed for the P388D.1 supernatants, medium conditioned by L929 cells (days 3 or 5) had significantly less protein ($P < 0.0001$) than the control group (Table 4-3). When compared over time (day 1 vs 5), there was ~45% decrease in the protein content of the cell-conditioned medium. Nonetheless, no linear correlation could be drawn between the total protein content and growth of *T. danilewskyi*. A duplicate experiment testing undiluted L929-conditioned medium (day 3 and 6) disclosed similar trends in parasite growth and total protein concentration (data not shown).

Trypanosoma danilewskyi was cultivated in CCL71 supernatants that were or were not diluted 50% in fresh medium. In all cases, conditioned supernatants that were diluted supported multiplication of trypanosomes better ($P < 0.002$) than undiluted samples (Table 4-5). Dilution did not markedly affect the ability of control medium to sustain trypanosomes. When compared to growth in control medium, the number of trypanosomes in wells containing supernatants (diluted) from CCL71 cultures, on days 6 and 9, increased nearly 40% ($P < 0.002$). Similar observations were made whether diluted or undiluted samples of conditioned or control medium were compared. As with supernatants from murine cell lines, a significant decrease ($P < 0.004$) in the protein concentration was observed in medium conditioned by CCL71 cells (Fig. 4-14).

DISCUSSION

Over 14 species of fish have been shown to be susceptible to *T. danilewskyi* [185, 303]. Common shiners, *Notropis cornutus,* brown bullheads, and carp developed lower parasitemia when compared to goldfish [81, 185, 303]. These observations suggest that genetically based factors and/or the immune status of the fish may be responsible for determining susceptibility to *T. danilewskyi*. The results of the present study suggest that the latter was the case, as medium supplemented with carp serum or GFS supported *T. danilewskyi* proportionately. Likewise, cultures containing tin foil barb serum had approximately 30% more parasites than those with GFS or carp serum did. While serum-related factors within piscine blood were requisite for cultivation of *T. danilewskyi*, it appeared as though blood products from some species were not appropriate for long-term cultivation. For example, *T. danilewskyi* could only be maintained 18 days in cultures containing 10% heat-inactivated rainbow trout plasma [14].

My findings are unique inasmuch as previous attempts to propagate the parasite in the presence of fish components necessitated the presence of living cells [251]. Additionally, my study indicated that the amount of piscine serum affects *in vitro* cultivation, as growth in 10% GFS was faster and greater than that in 5% FBS and 5% GFS. Correspondingly, other workers have since reported that suboptimal growth of *T. danilewskyi* was obtained with as little as 1% fish serum and 9% FBS [216].

Trypanosome cultures containing 10% GFS were shown to contain primarily bloodstream forms (60 to 80%). Inasmuch as *T. danilewskyi* was commonly maintained in goldfish by inoculation with infected blood [183, 299], this morphometric study inferred that >80% of the cultured trypanosomes were able to establish a patent infection. Further, this finding confirmed the feasibility of cultured trypanosomes as a source of antigen used in assays that tested the immune response of trypanosome-infected fish. As reported by Overath *et al.* [216], *T. danilewskyi* cultured in 5% fish serum and 5% FBS were indistinguishable from those derived from fish blood. For instance: 1) both have a bloodstream-form morphology; 2) cultured or fish trypanosomes were recognized by a monoclonal antibody directed against a surface epitope; and 3) ultrastructurally, the surface of both forms have a fuzzy carbohydrate-rich surface coat.

A small percentage of the parasite population, cultured in 10% GFS, consisted of morphological forms that would be found in the leech. With the exception of metacyclic trypomastigotes, the difference in these forms on different culture days were negligible. As the cultures became older, the percentage of metacyclic trypomastigotes increased noticeably. The latter observation was consistent with that reported for mammalian trypanosome cultures. Namely, as the culture attained its final phase of exponential growth, the number of metacyclic-forms increased steadily, reaching 25% of the population. The appearance of metacyclic trypomastigotes in the leech correlated directly with the digestive phases. If digestion goes on more slowly, the appearance of metacyclic-forms in the proboscis sheath were correspondingly delayed [230]. With regard to nutrient availability and the accumulation of parasite-released metabolic products, the older cultures may reflect a crude comparison with the crop of the leech preceding a blood meal. While this hypothesis offers a possible explanation for the increase in the number of metacyclic-forms, it is clear that numerous exogenous factors (i.e., temperature, uric acid, proteolytic products of haemoglobin) affected differentiation [65, 104, 232].

Medium supplemented with mammalian serum (10% FBS or horse serum) did not support the growth of *T. danilewskyi*. These findings were consistent with those of Overath *et al.* [216] who postulated that 10% FBS alone has a toxic effect on *T. danilewskyi*. Further, *T. danilewskyi* did not multiply in Hank's solution supplemented with 10% FBS, SNB9 with human or sheep blood, or SNB9 with sheep blood and various vitamins [141]. The suitability of mammalian sera for the cultivation of *T. danilewskyi* remains in question, because: 1) L4NHS medium (consisting of basal broth, rabbit serum, and erythrocyte lysate from rabbit blood) facilitated exponential growth and indefinite culturing [233]; and 2) minimal essential medium supplemented with Hank's salts, L-glutamine, and 25% FBS yielded a significant increase in the number of trypanosomes (9 to 15 days postincubation) [14].

Serum-free medium failed to maintain trypanosomes that were isolated from experimentally infected goldfish. Using a different isolate of *T. danilewskyi* (TsCc-NEM), corroborative evidence was published more recently [216]. Collectively, these findings were markedly different from a report that showed serum-free medium to support both short- and long-term cultivation of the haemoflagellates [284]. While numerous factors may account for this difference, I believe that the strain adapted in Dr. Belosevic's laboratory (> 48 passages) for growth in serum-free medium succumbed to prolonged cryopreservation. Evidently, serum-free media (GFLM or Insect Xpress) does not contain the components necessary for growth of the surviving trypanosomes.

Although seemingly counterintuitive, histopathological studies suggest that haemopoietic organs provided a suitable environment for *T. danilewskyi*, as 8 days after inoculation of the infective stage, a large number of trypanosomes were located extravascularly in the kidneys and spleen. By day 14, aggregates of *T. danilewskyi* fill the kidneys [81]. Experiments using Transwell cell culture inserts or cell-conditioned supernatants supported this hypothesis and suggest: 1) cell to cell contact was not required for growth enhancement; and 2) parasite growth was related to the number of kidney leukocytes seeded in the culture. As well, a greater number of trypanosomes was observed in those wells containing medium obtained from older macrophage cultures.

At present, it is unclear if growth-enhancing activity of macrophages for mammalian trypanosomes is associated with the host's immune response. Viens *et al.* [277] reported that peritoneal cells, isolated from an immune mouse, support growth of *T. musculi* as well as those acquired from a normal mouse. Conversely, other studies [7, 116] demonstrated that splenic macrophages obtained from previously infected animals do not support trypanosome cultures when compared to those from normal donors. Observations in the latter studies may imply the presence of a macrophage memory population. Alternatively, the inhibition of trypanosome growth may be attributed to the presence of residual lymphocytes in the primary cell cultures. My experiments, using cell-free supernatants, suggest that supportive activity was independent of the course of infection, as parasite growth in medium collected from cultures derived from infected fish did not differ markedly from those acquired from normal fish.

Species specificity of growth-enhancing activity was lacking, inasmuch as *in vitro* growth of *T. danilewskyi* could be augmented by medium conditioned by human and mouse cells. These conclusions were consistent with those of investigators who showed that rat [8, 277, 278] and human cells [278] could support growth of mouse trypanosomes. Collectively, these findings suggested that trypanosomes could use molecules that have been conserved between species.

Several species of trypanosomes have been successfully cultivated in fibroblast-like cells [1, 26, 83, 266, 272]. In some cases, trypanosome growth continued even after the fibroblast feeder layer degenerated, as long as medium was changed regularly [26, 272]. Because many products of fibroblasts were also produced by macrophages (i.e., interleukin-6, interleukin-8, interferon-α, interferon-β, macrophage chemotactic proteins, and platelet-derived growth factor [74]), I tested if the activity of conditioned medium was limited to macrophages. The results indicated that growth-enhancing activity was not specific to cell-type, as medium conditioned by mouse or goldfish fibroblasts augmented growth of *T. danilewskyi*. Nevertheless, this did not imply that the same factor(s) was responsible for the increase in parasite numbers.

In certain experiments, diluted cell-conditioned medium facilitated parasite growth better than undiluted samples. This observation may reflect the generation time of the purchased cell lines. For instance, at the stated cell density, U937 cells require passaging every second day. Culturing beyond the second day results in increased acidity, marked decrease in total protein content, presumably a build-up of toxic byproducts, and eventually death of the monocytes. It was possible that *T. danilewskyi* cannot grow in medium conditioned by U937 monocytes for 3 or 5 days, notwithstanding the presence of growth-enhancing factors, because of adverse environmental conditions. Alternatively, increased quantities of growth enhancing factors may have differential effects on *T. danilewskyi*.

At present it is unknown what these growth enhancing factors were and by what mechanism they facilitated growth of *T. danilewskyi*. There was some indication that the active substance was required in small quantities, as cell-conditioned media enhanced parasite growth notwithstanding the decreases in total protein content. Alternatively, the growth-enhancing factor may be something other than a protein. For example, numerous investigators have attributed trypanosome growth to carbohydrates [118, 252], minerals [16, 164], purine bases [32, 120], and lipids [37, 61].

Table 4-1. Effect of serum-free medium (GFLM) and horse serum on *Trypanosoma danilewskyi* cultivated *in vitro*[a].

TIME (week)	Number of parasites ml^{-1} X (10^{-3})	
	10% Horse serum	GFLM
1	64.4 ± 5.28[b]	12.7 ± 2.82
2	41.2 ± 8.50	2.9 ± 1.39
3	34.9 ± 4.49	0.4 ± 0.18
4	32.1 ± 6.75	0.1 ± 0.05
5	17.4 ± 2.06	0
6	20.3 ± 2.90	0

[a] each flask was initially inoculated with 6.7 X 10^{-4} parasites ml^{-1} medium

[b] expressed as the mean number of parasites ± SEM (n = 6)

Table 4-2. *In vitro* growth of *Trypanosoma danilewskyi*[a] in Transwell cell culture inserts containing kidney leukocytes derived from goldfish.

	No cells	With cells	
TIME (day)		10^5 cells	10^6 cells
4	6.8 ± 0.01[b]	7.1 ± 0.05[c]	7.2 ± 0.02[c]
18	6.4 ± 0.13	6.7 ± 0.02[c]	7.1 ± 0.01[c,d]

[a] each well was initially inoculated with 1 X 10^5 parasites

[b] mean number of parasites ($\log_{10}$) ± SEM (n = 2 or 3)

[c] $P < 0.01$ when compared on same day to control group

[d] $P < 0.001$ when compared on same day to wells containing 10^5 cells well^{-1}

Table 4-3. Protein concentration of undiluted supernatants collected from mammalian macrophage (P388D.1) or fibroblast (L929) cell-lines.

Supernatant age (day)	Protein concentration			
	Control	Macrophage	Control	Fibroblast
1	42.5 ± 0.34[a]	41.3 ± 0.42[c]	44.2 ± 0.54	44.2 ± 0.87[c]
3	43.3 ± 0.72[b]	24.7 ± 0.62[c]	44.3 ± 0.33[b]	37.3 ± 0.72[c]
5	43.5 ± 0.56[b]	14.7 ± 0.33	44.2 ± 0.70[b]	23.8 ± 1.24

[a] data expressed as the mean protein concentration ($\mu g\ \mu l^{-1}$) ± SEM (n = 6)
[b] $P < 0.0001$ when compared on same day to conditioned medium
[c] $P < 0.0001$ when compared to day 3 or 5 in the same group

Table 4-4. *In vitro* growth of *Trypanosoma danilewskyi*[a] in control medium or medium conditioned by mouse fibroblasts, L929.

Supernatant age (day)	Control medium		Conditioned medium	
	Undiluted	Diluted 50%	Undiluted	Diluted 50%
1	7.7 ± 0.34[b]	6.9 ± 0.10	57.4 ± 1.11[d]	48.8 ± 2.24[c,d]
3	8.2 ± 0.37	6.8 ± 0.16[c]	22.7 ± 1.28[d]	92.6 ± 4.96[c,d]
5	8.2 ± 0.30	7.5 ± 0.07	19.7 ± 1.88[d]	109.7 ± 7.34[c,d]

[a] each well was initially inoculated with 1 X 10^5 parasites

[b] data expressed as mean number of parasites X 10^{-4} ± SEM (n = 6)

[c] $P < 0.01$ when diluted sample compared to undiluted sample within same treatment

[d] $P < 0.0001$ when similar dilutions are compared between treatment groups

Table 4-5. *In vitro* growth of *Trypanosoma danilewskyi*[a] in control medium or medium conditioned by fish fibroblasts, CCL71.

Supernatant age (day)	Control medium		Conditioned medium	
	Undiluted	Diluted 50%	Undiluted	Diluted 50%
6	30.8 ± 0.42[b]	32.3 ± 1.20	47.5 ± 0.52[d]	51.8 ± 1.23[c,d]
9	28.8 ± 1.01	32.2 ± 0.39	33.2 ± 0.80[d]	54.3 ± 1.22[c,d]

[a] each well was initially inoculated with 1 X 10^5 parasites

[b] data expressed as mean number of parasites (X 10^{-4}) ± SEM (n = 5)

[c] $P < 0.002$ when diluted sample compared to undiluted sample within same treatment

[d] $P < 0.002$ when similar dilutions are compared between treatment groups

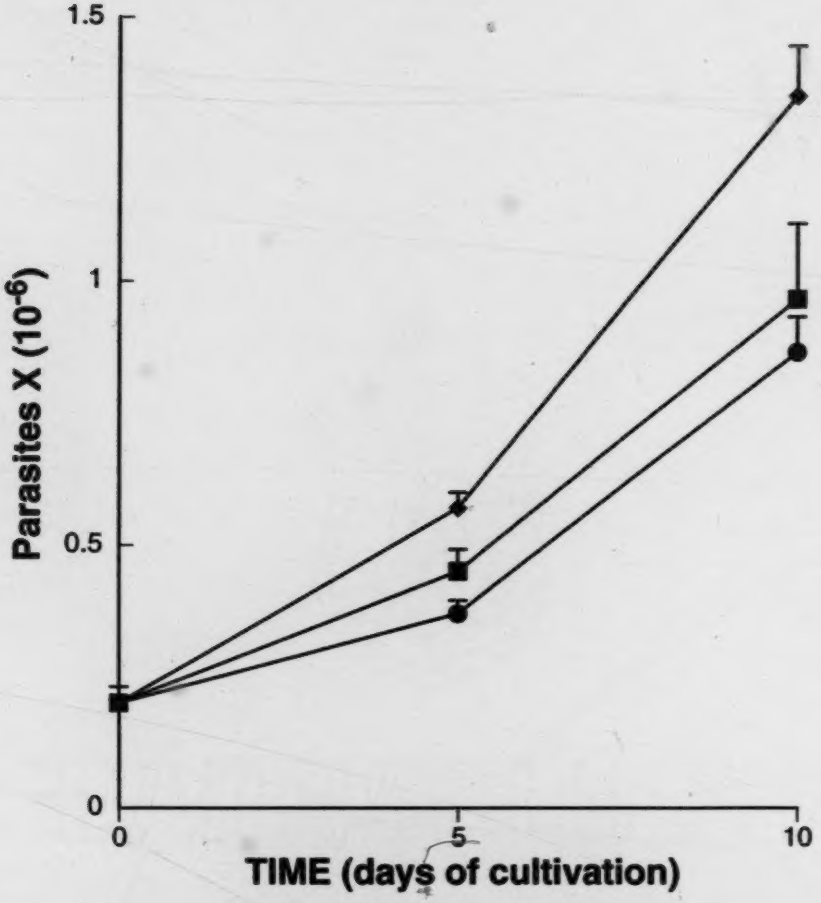

Fig. 4-1. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium supplemented with ■ 10% goldfish, ● 10% carp, or ◆ 10% tin foil barb serum. Trypanosomes were seeded into 25 cm^2 flasks at a concentration of approximately 2 X 10^5 ml^{-1}. Data points represent the mean number of parasites ml^{-1} medium ± SEM (n = 4).

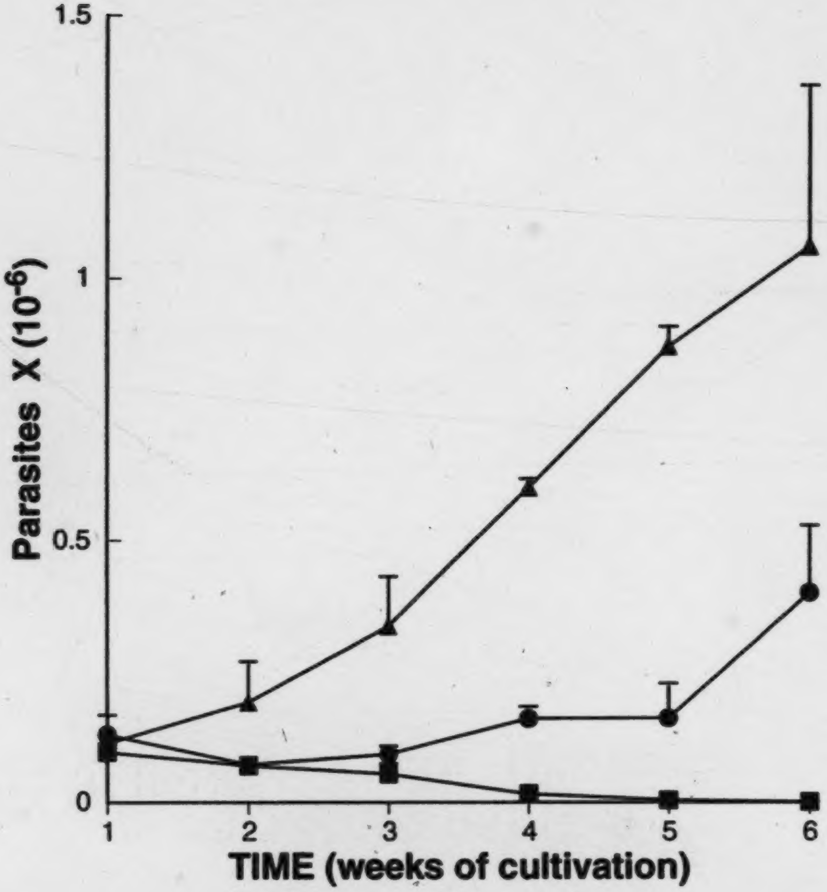

Fig. 4-2. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium supplemented with ■ 10% foetal bovine serum (FBS), ▲ 10% goldfish serum (GFS), or ● 5% FBS and 5% GFS. Trypanosomes were seeded into 75 cm^2 flasks at a concentration of approximately 6.7 X 10^4 ml^{-1}. Bar height represents the mean number of parasites ml^{-1} of medium ± SE (n = 6).

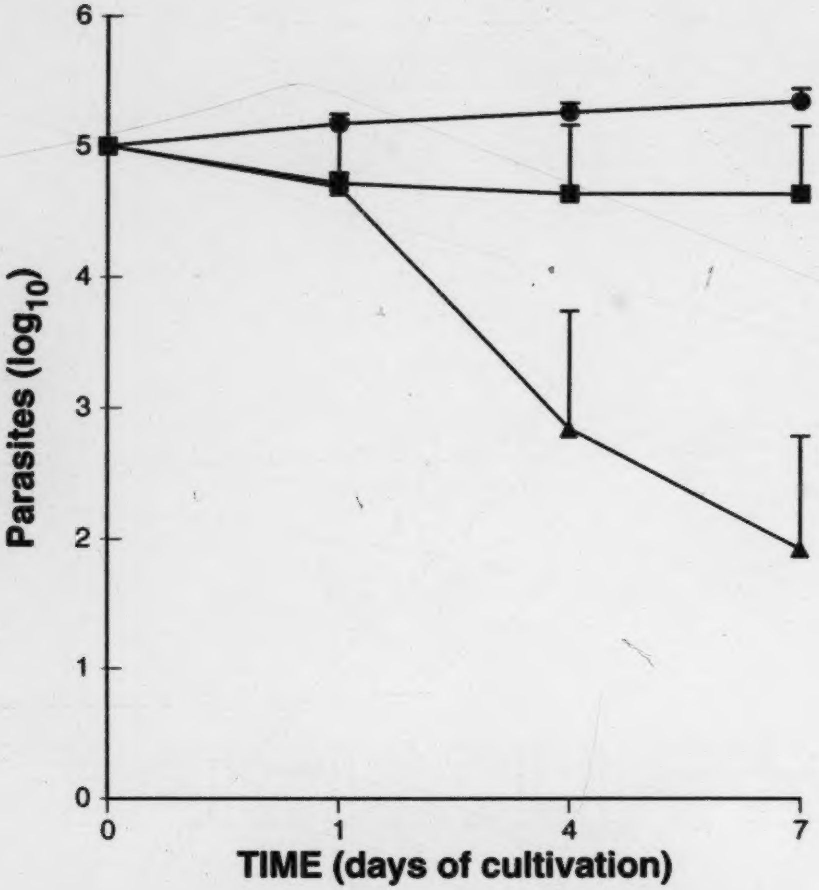

Fig. 4-3. *In vitro* growth of *Trypanosoma danilewskyi* cultured in ▲ serum-free medium or sera collected from ■ infected (75 dpi) or ● naïve fish. Trypanosomes were seeded into a 96-well plate at a concentration of approximately 1 X 10^5 well^{-1}. Data points represent the mean number of parasites ± SEM ($n \geq 6$).

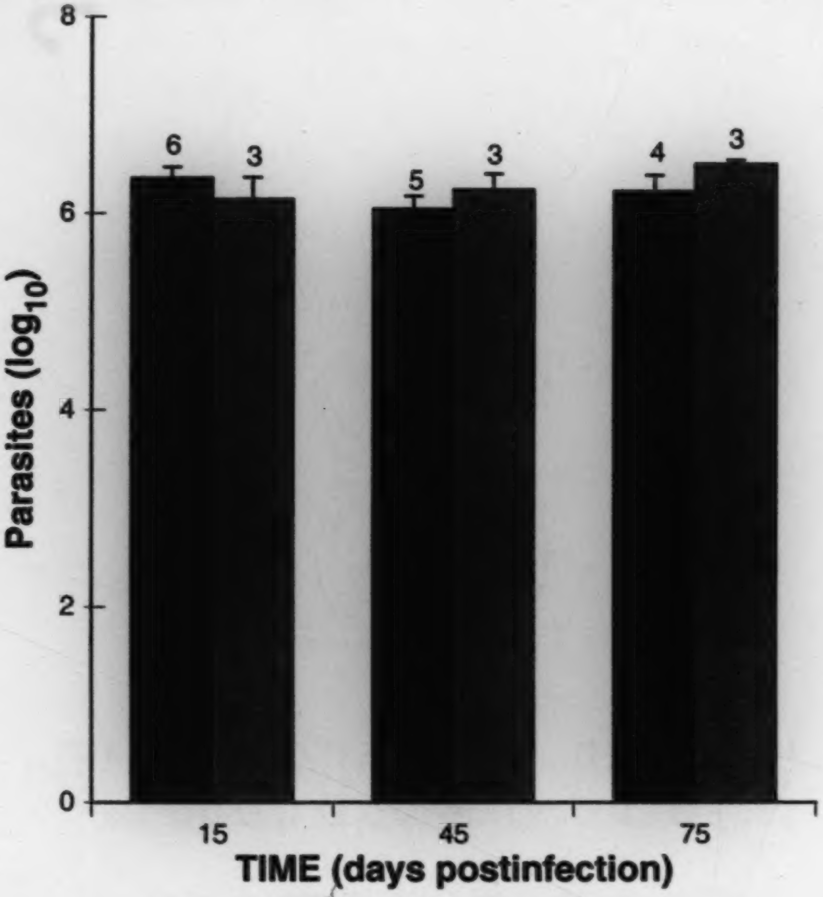

Fig. 4-4. *In vitro* growth of *Trypanosoma danilewskyi* cultured in 10% sera collected from infected (black bar) or naïve goldfish (gray bar). Trypanosomes were seeded into a 96-well plate at a concentration of approximately 1 X 10^5 $well^{-1}$. Bar height illustrated the mean number of parasites ± SEM. Sample size is indicated above each bar.

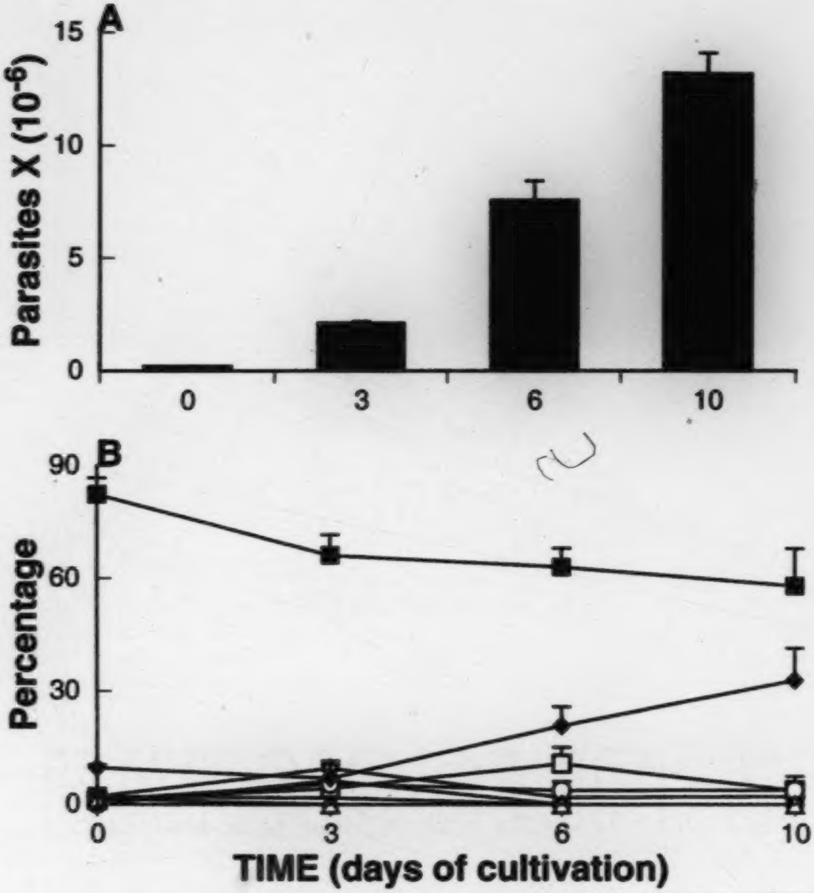

Fig. 4-5. *In vitro* growth (A) and morphological forms (B) of *Trypanosoma danilewskyi* present in medium supplemented with 10% goldfish serum. Bar height (A) represents the mean number of parasites ml^{-1} medium ± SEM (n = 6). Data points (B) represent the mean percentage ± SEM of the morphological form present in the cultures. ■ Bloodstream-form, ● stumpy-form, ▲ crithidial, ✧ dividing-form, ☐ leptomonad, ○ slender-form, Δ trypanoform, and ◆ metacyclic trypomastigotes.

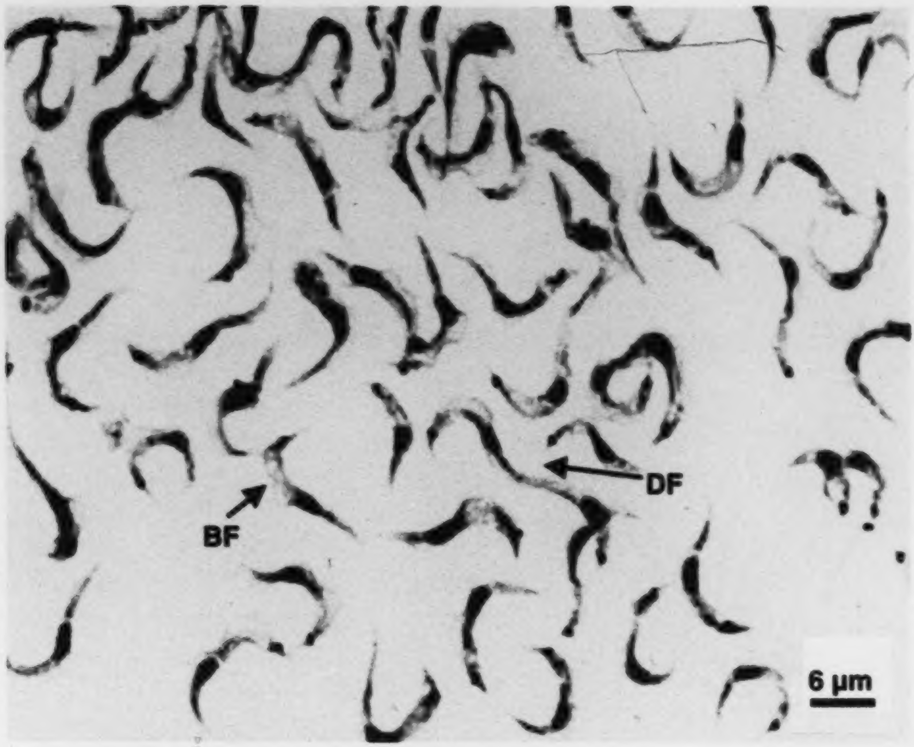

Fig. 4-6. Wright's-stained smear of *Trypanosoma danilewskyi* cultured for 6 days in goldfish leukocyte medium supplemented with 10% goldfish serum. Bloodstream form and dividing form indicated by BF and DF, respectively.

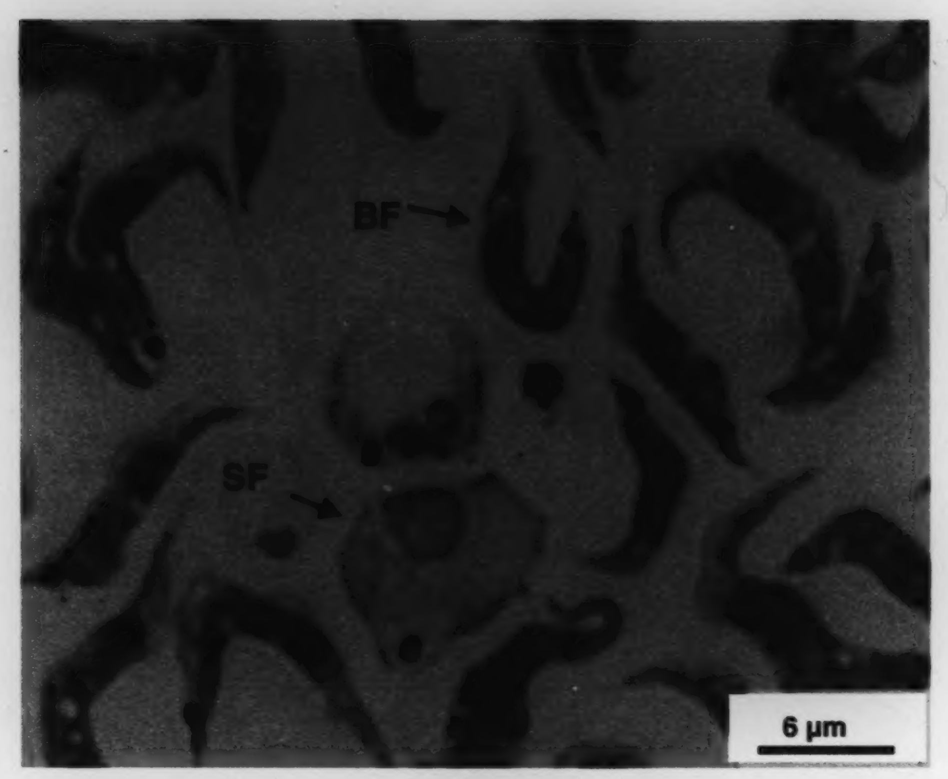

Fig. 4-7. Wright's-stained smear of *Trypanosoma danilewskyi* cultured for 6 days in goldfish leukocyte medium supplemented with 10% goldfish serum. Bloodstream form and stumpy form are indicated by BF and SF, respectively.

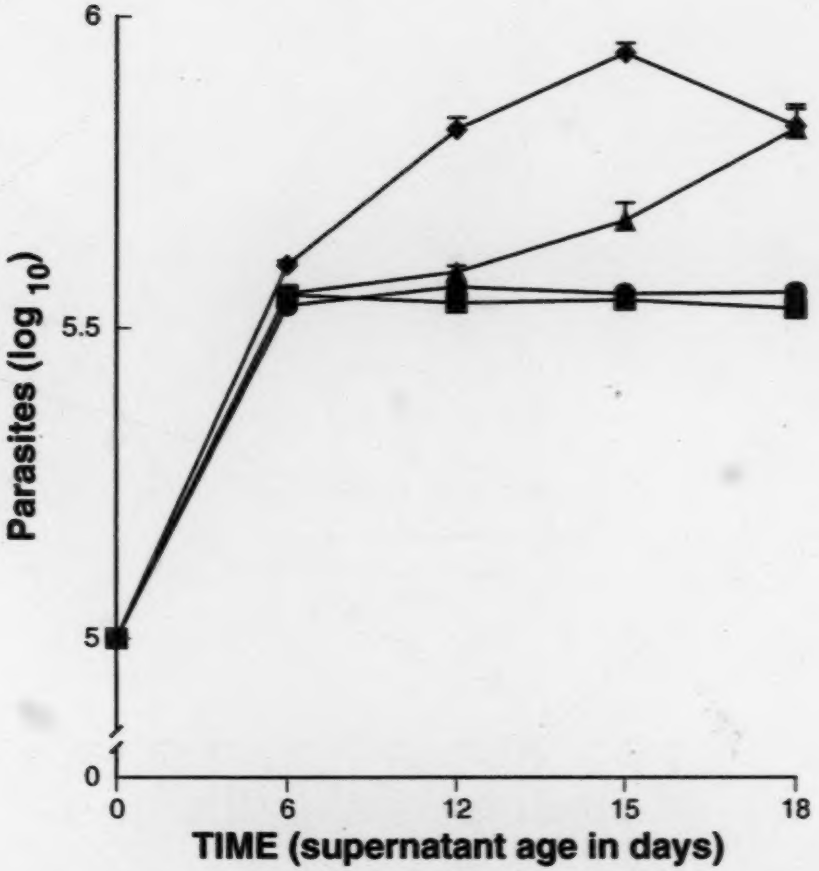

Fig. 4-8. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium conditioned by ◆ 3 X 10^5, ▲ 10^5, ● 10^4, or ■ no fish macrophages ml^{-1}. Data points represent the mean number of parasites ± SEM (n = 6).

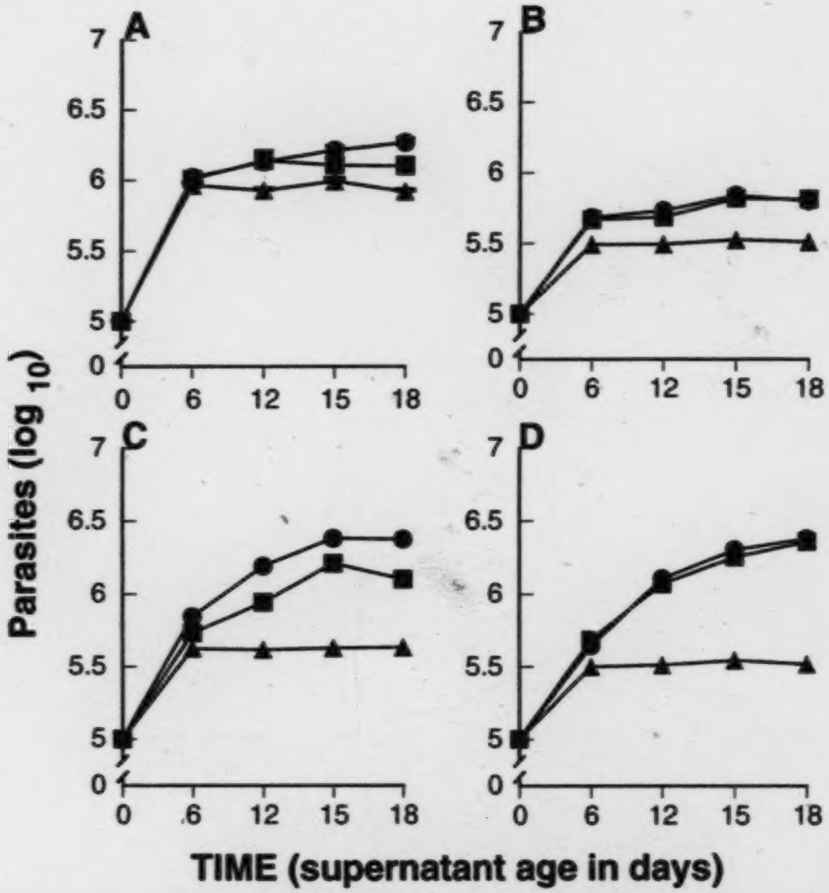

Fig. 4-9. *In vitro* growth of *Trypanosoma danilewskyi* cultured in ▲ control medium or medium conditioned by macrophages derived from ● uninfected or ■ infected (A, 28 dpi; B, 56 dpi: C, 112 dpi: D, 152 dpi) fish. Trypanosomes were seeded into a 96-well plate at a concentration of approximately 1 X 10^5 well^{-1}. Data points represent the mean number of parasites ± SEM (n = 5).

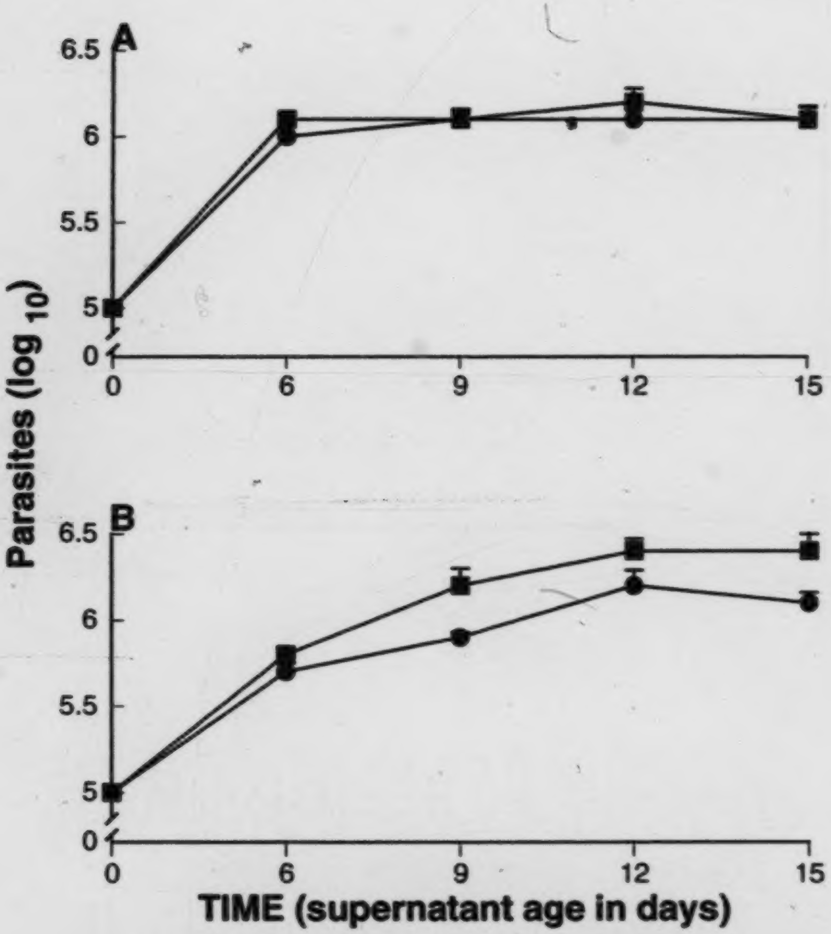

Fig. 4-10. *In vitro* growth of *Trypanosoma danilewskyi* cultured in medium conditioned by macrophages derived from ● uninfected or ■ infected (A, 70 dpi; B, 112 dpi) fish. Trypanosomes were seeded into a 96-well plate at a concentration of approximately 1 X 10^5 well^{-1}. Data points represent the mean number of parasites ± SEM (n = 2 or 3).

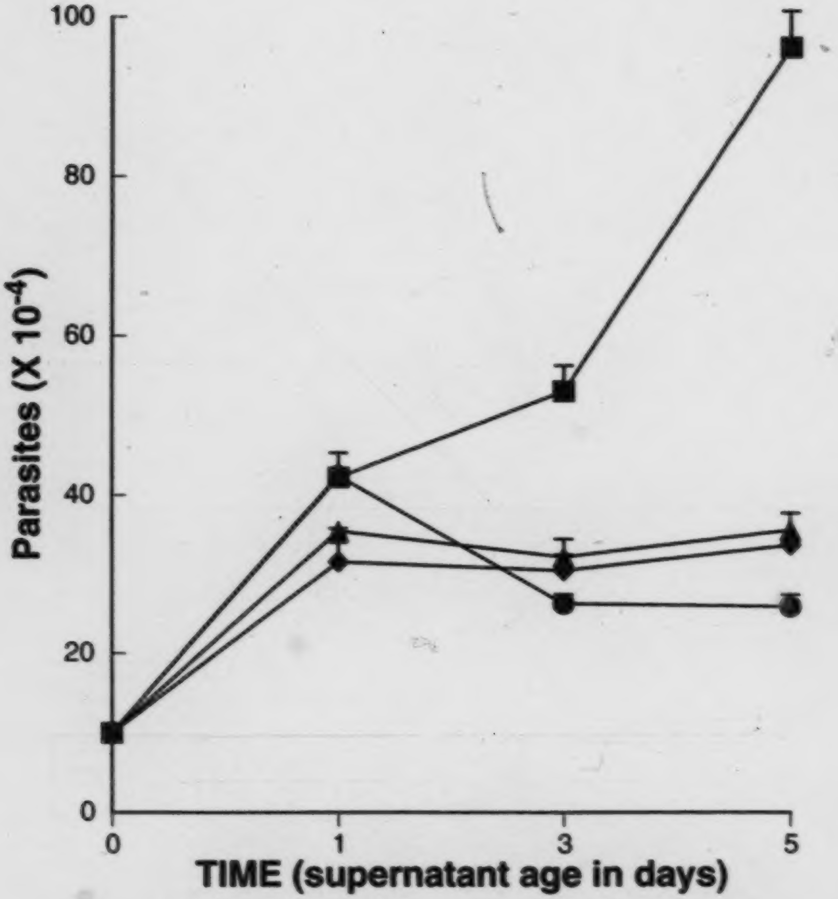

Fig. 4-11. *In vitro* growth of *Trypanosoma danilewskyi* cultured in (◆ 100% or ▲ diluted in fresh medium 50% v/v) control medium or (● 100% or ■ diluted in fresh medium 50% v/v) medium conditioned by human monocytes, U937. Trypanosomes were seeded into a 96-well plate at a concentration of approximately 1 X 10^5 well^{-1}. Data points represent the mean number of parasites ± SEM (n = 6).

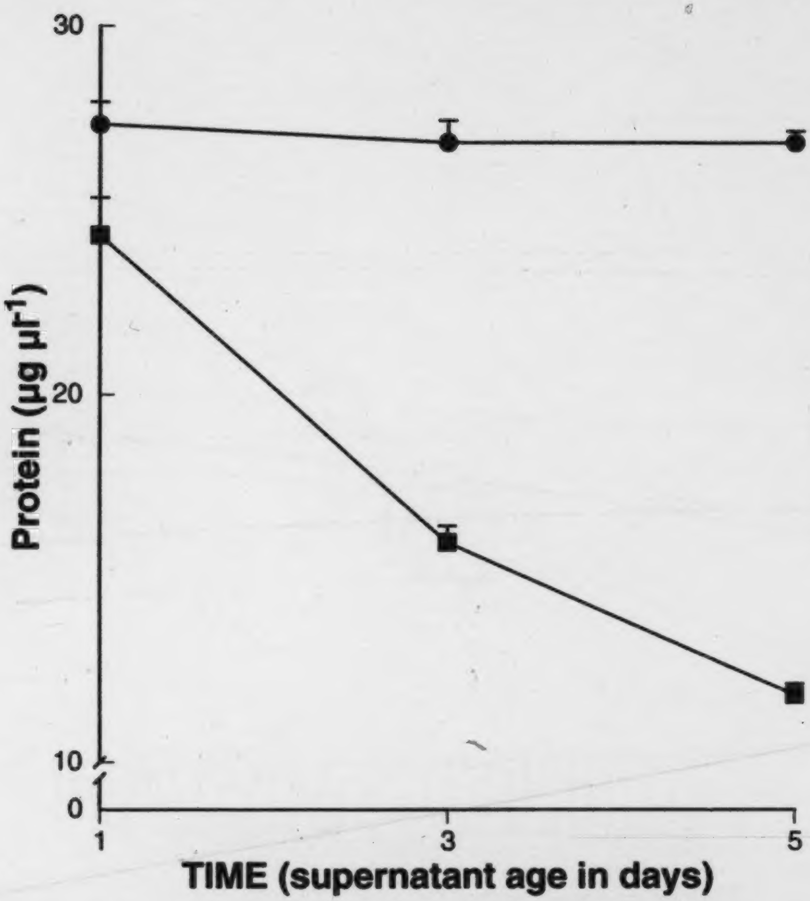

Fig. 4-12. Protein concentration of undiluted ● control medium or ■ medium conditioned by human monocytes, U937. Data points represent the mean protein concentration ± SEM (n = 6).

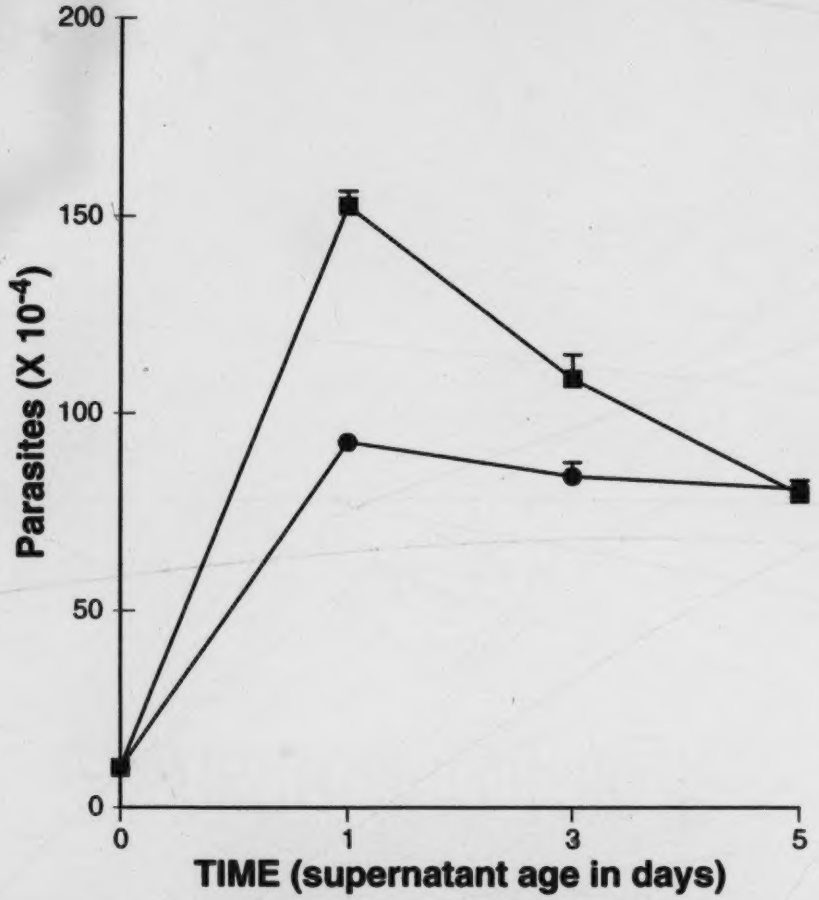

Fig. 4-13. *In vitro* growth of *Trypanosoma danilewskyi* cultured in ● control medium or ■ medium conditioned by murine macrophages, P388D.1. Trypanosomes were seeded into a 96-well plate at a concentration of approximately 1 X 10^5 well^{-1}. Data points represent the mean number of parasites ± SEM (n = 6).

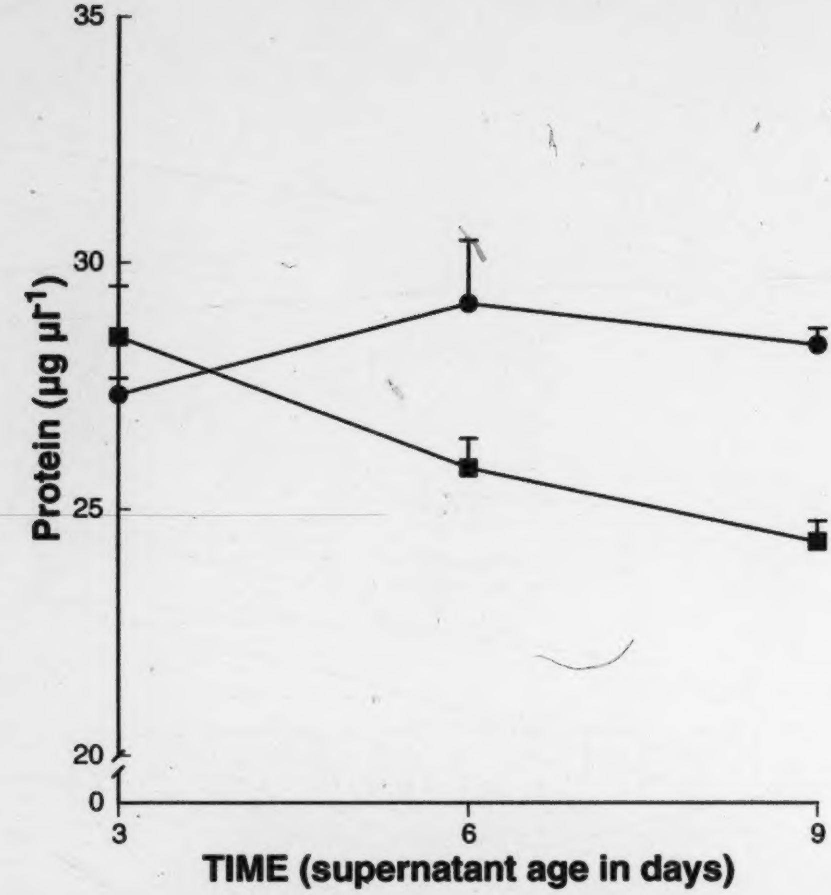

Fig. 4-14. Protein concentration of undiluted ● control medium or ■ medium conditioned by fish fibroblasts, CCL71. Data points represent the mean protein concentration ± SEM (n = 6).

CHAPTER V

ANALYSIS OF ACTIVE IMMUNIZATION OF GOLDFISH FOR PROTECTION AGAINST *TRYPANOSOMA DANILEWSKYI*

INTRODUCTION

The majority of fish that survive a primary *T. danilewskyi* infection develop resistance to reinfection. The protection for subsequent challenge in the surviving fish has been reported to be at least 89 [140], 190 [298], and 365 [283] days after the primary infection. This state of resistance was believed to result from non-sterile immunity [140, 186]. Inasmuch as the rate of infection and infection dynamics are affected by the physiological status of the host and environmental factors [141, 309], fish may carry an infection for a prolonged period, even years [186]. Based on these findings, a reasonable hypothesis is that resistance to secondary *T. danilewskyi* infection is immunologically mediated. Further, parasite antigens, accessible to the immune system during a primary infection, can be isolated from cultured trypanosomes and used for stimulating a blastogenic response and immunity. An unpublished study [185] indicated that a protective response could not be evoked in goldfish by successive immunization with thermally killed trypanosomes. Notwithstanding, a comprehensive study was warranted to determine if resistance could be conferred by immunization with other parasite fractions.

The main objective of experiments reported in this chapter was to screen different types of antigenic preparations (sodium perchlorate-killed parasites, parasite lysate, water-soluble molecules, detergent-solubilized molecules, and ES products) and to determine their effects on the susceptibility of goldfish to *T. danilewskyi*. Further, a preliminary experiment was conducted *in vitro* to determine whether leukocytes, obtained from recovered fish, were responsive to *T. danilewskyi* antigen. Additionally, a study was conducted to provide confirmatory evidence of passive transfer of immunity to naïve fish by inoculation of serum from an immune donor. Parameters used to assess the efficacy of immunization included: 1) mortality; 2) prevalence of infection; 3) abundance of parasites; 4) red cell volume; and 5) presence of parasite-specific antibodies.

EXPERIMENTAL DESIGN

Active immunization

Before immunization, blood was analyzed using the haematocrit centrifuge technique [296] to ensure that fish were free of trypanosomes. Fish (9.5 to 11 cm standard length) were inoculated with sodium perchlorate-treated parasites, parasite lysate, ES products, detergent-solubilized molecules, or water-soluble molecules. Parasite extracts from 3 X 10^7 organisms or the appropriate control solution was administered to the fish intravenously, intramuscularly, or intraperitoneally. Similarly, extracts or control solution were administered intraperitoneally in an equal volume (70 µl) of FIA or FCA. Two separate groups of fish served as non-immunized controls (i.e., non-immunized infected and non-immunized uninfected). Each treatment group was comprised of five or six fish. Thirty days after immunization, fish were inoculated intraperitoneally with approximately 6.25 X 10^6 *T. danilewskyi* or an equal volume of GFLM. On day 0, 4, 7, 14, 28, and 56 postinfection, the success of each treatment was determined by enumerating the number of parasites present in the blood of immunized and control fish. Further, these experiments determined whether the red cell volume and the humoral response of the fish were related to the magnitude of protection.

Two precautions were taken to ensure that immunization with parasite extract did not cause an infection that would consequently confer resistance. First, parasite extract, equivalent to 3 X 10^7 trypanosomes, was inoculated into flasks (25 cm^2) containing GFLM, 10% FBS, 5% GFS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and/or medium conditioned by fish kidney leukocytes [36]. These flasks were cultured for 25 days at 20°C and examined visually (200 X) every 3 or 4 days for the presence of parasites. Secondly, blood, collected from fish 30 days post-vaccination, was examined (using the haematocrit centrifuge technique [296]) to ensure that goldfish were free of *T. danilewskyi*.

Antigen-induced proliferation of leukocytes

A preliminary study was conducted to determine the antigen-induced responsiveness of PBL isolated from *T. danilewskyi*-infected fish (112 dpi). Optimal concentration of reagents was determined using criss-cross serial dilution analyses. Aliquots of 0, 10^5, and 10^6 PBL, derived from immune or naïve fish, were seeded into wells (96-well plate) containing ~25% GFS and trypanosome lysate (0, 1, 10, or 100 µg protein). After 5 days of incubation at 20°C, the number of cells in individual wells was estimated using the XTT tetrazolium salt viability assay.

Passive transfer of immunity

For the passive transfer immunization experiment, fish (14 to 18 cm standard length) were immunized intravenously with 150 µl of immune serum, collected from donors 245 dpi, or nonimmune serum. Twenty-four hours after immunization, all fish were inoculated intraperitoneally with approximately 6.25 X 10^6 trypanosomes. A third group of fish served as non-immunized infected controls. Blood was collected from each fish 0, 7, 14, and 21 dpi to monitor parasitemia.

RESULTS

Active immunization

Results from two representative experiments (i.e., administration of sodium perchlorate-treated parasites or trypanosome ES products) are presented in this section. In a similar format, the results from the remaining three experiments are presented in Appendix B. Nevertheless, because some concepts are only demonstrated in the latter experiments, the discussion section contains summary tables and text relevant to all experiments.

Excretory/secretory products

Intravenous administration. Intravenous immunization with ES products was seemingly inconsequential to the outcome of the challenge infection. Although all fish were infected with *T. danilewskyi* by 7 dpi, no mortality occurred in the immunized or control group (Fig. 5-1A). The prevalence of infection in the immunized group was identical to that observed in the control group (Fig. 5-1B). Briefly, 7, 14, and 28 dpi, trypanosomes were found in the blood of all fish. By day 56, parasites were detected in 60% of the fish. Although the mean parasite intensity of the immunized fish was higher than that in the control group (7, 14, and 28 dpi), no significant differences were observed when the parasitemia of the two groups were compared (Fig. 5-1C). When examining the parasitemia of individual fish, that were immunized intravenously, it was difficult to conclude if the inoculation procedure was successful in all immunized fish. For example, three of the five fish inoculated with ES products had parasitemias that were comparable to the control fish ($\leq 7.5 \times 10^5$ parasites ml^{-1} blood). The two other experimental fish had infections that exceeded 2×10^6 trypanosomes ml^{-1} blood.

The antibody response of fish was not significantly affected by intravenous immunization with ES products or *T. danilewskyi* infection, as the mean ELISA values of each treatment group was comparable to that observed 0 dpi (Fig. 5-1E). Although comparisons between the two groups were not significant, the percent of control value suggested that the antibody response of immunized fish, during the first 28 days of the infection, increased slightly more than that of the fish inoculated intravenously with control solution (Table 5-1).

No significant correlation was drawn between the parasitemia of immunized or control fish and the ELISA values attained from sera reacted against ES products (Table 5-2). Examination of data of individual fish demonstrating a marked increase (two-fold) in

ELISA values was only observed in an immunized fish with parasitemias exceeding 1 X 10^7 ml^{-1} blood. In comparison, the immunized fish with the lowest parasitemia (< 1500 parasites ml^{-1} blood) had ELISA values that were less than or equal to that observed 0 dpi (data not shown).

The red cell volumes of fish, inoculated with ES products or control solution, followed similar patterns. For this reason, results relevant to this experiment will be presented once in this subsection. During the first week of *T. danilewskyi* infection, the red cell volumes of fish inoculated with ES products (i.e., all administration routes) were not significantly different from those of the respective control group (Fig. 5-1D to 5-5D). Later in the infection, the only difference between immunized and control fish was observed in the fish inoculated intraperitoneally with FCA (Fig. 5-5D). The red cell volumes of fish given intraperitoneal inoculations (without adjuvant or with FCA) did not differ significantly over time (Fig. 5-3D and 5-5D). Conversely, the mean red cell volumes of fish inoculated intravenously, intramuscularly, or intraperitoneally (with FIA) differed significantly (Fig. 5-1D, 5-2D, and 5-4D). In these instances, the mean value on 28 and 56 dpi were higher ($P < 0.03$) than that observed on 14 dpi. During the first week of infection, the red cell volumes observed in the non-immunized infected group was significantly higher ($P < 0.04$) than that in the non-immunized uninfected group (Fig. 5-7D). In both groups, the lowest value of approximately 30% was recorded on 14 dpi. Thereafter, the mean red cell volume in these fish increased significantly ($P < 0.01$).

Intramuscular administration. Under the described conditions, the response of fish to *T. danilewskyi* was modestly affected by intramuscular administration of ES products. While all of the fish in the immunized group survived, one of the five control fish died with massive parasitemia (Fig. 5-2A). As observed previously, all of the control fish became infected within 1 wk of the challenge inoculum (Fig. 5-2B). In contrast, the prevalence of infection in the immunized group reached a maximum of 80% by 14 dpi. Parasitemia of immunized fish (7, 14, and 28 dpi) was approximately two common logarithms lower than that observed in the control group (Fig. 5-2C). However, because of the immense variability between individual fish in the same group, this comparison was not significant. Fifty-six days after the challenge inoculum, the prevalence of infection and parasite abundance of the experimental and control groups was comparable (Fig. 5-2B and 5-2C).

Comparison of mean ELISA values on 0 dpi indicated that intramuscular administration of ES products did not affect the humoral response significantly (Fig. 5-2E). However, during the course of infection, optical density values of sera obtained from fish inoculated intramuscularly with control solution, were distinctly higher than that observed in the immunized group. Although mean ELISA values of control fish were consistently higher than in the immunized group, the greatest difference (approximately two-fold) was observed 28 dpi ($P < 0.05$). Similarly, the lowest percent of control value (56%) was observed 28 dpi (Table 5-1).

ELISA values of samples, collected from control fish with a maximum parasitemia of 1×10^6 trypanosomes ml^{-1} blood, never exceeded 20% above that on 0 dpi. In comparison, samples from the remaining control fish, with 3×10^8 or 1×10^9 trypanosomes ml^{-1} blood, had ELISA values that exceeded that observed on 0 dpi by 50 and 190%, respectively (data not shown). Further, optical densities correlated ($r = 0.70$) with the parasitemia of individual control fish (Table 5-2). Although an insignificant positive correlation between parasitemia and ELISA values was observed in immunized fish, similar trends were observed. A noteworthy example was of an immunized fish that never developed a patent infection. Suppression of the humoral response seemed to be greatest in this fish, as ELISA values (28 and 56 dpi) were approximately 40% lower than that observed 0 dpi (data not shown).

Intraperitoneal administration (no adjuvant). Although no mortality occurred in the immunized or control group (Fig. 5-3A), intraperitoneal administration of ES products had a favorable effect on the ability of fish to respond to the challenge infection. On 7 and

14 dpi, *T. danilewskyi* was detected in the blood of all control fish (Fig. 5-3B). As time progressed, two of these fish had low parasitemias such that parasites were detected intermittently. The maximum prevalence of infection (60%) was detected in immunized fish on day 14 and 28. When comparing experimental and control fish, there was no significant difference in the abundance of parasites (Fig. 5-3C). However, based on the prevalence of infection and parasitemias, the response of experimental fish can be separated into two distinct groups. For example, three of the five fish had no or very low numbers (< 1500 trypanosomes ml^{-1} blood) of parasites whereas the other two experimental fish had a course of infection that was similar to the control fish. As observed previously, sera from immunized fish with no or very low parasitemia (< 1500 ml^{-1} blood) for the duration of the experiment had ELISA values that were consistently below that recorded for 0 dpi. Conversely, parasitemias exceeding 1×10^6 ml^{-1} blood was associated with an increase of at least 20% in the optical density (data not shown). Correspondingly, a positive correlation ($P < 0.02$) between parasitemia and ELISA values were observed in the immunized group (Table 5-2). Although a positive correlation ($r = 0.32$) between parasitemia and the antibody response was observed in samples collected from control fish, some aberrations from the described trend were observed. For instance, serum from one fish with low parasitemia (< 8000 parasites ml^{-1} blood) had ELISA values that exceeded that on day 0 by 75% (56 dpi). Also, serum from a fish with > 2.5×10^7 trypanosomes ml^{-1} blood had only a 15% increase in the optical density (data not shown).

Prior to *T. danilewskyi* infection, the mean ELISA value of intraperitoneally immunized fish was not significantly different from that observed in the control group (Fig. 5-3E). Likewise, no statistical differences between the immunized and control group were detected during the course of infection. For each day of observation, the percent of control value remained below 100% (Table 5-1). The latter observation indicated that immunization does not facilitate the production of anti-trypanosome antibodies as well as control fish given a primary infection.

Intraperitoneal administration (FIA). Although some trends were evident, a significant level of protection against *T. danilewskyi* was not conferred by administration of ES products with FIA. All immunized and control fish survived the challenge infection (Fig. 5-4A). By 14 dpi, all control fish had developed a patent infection, whereas the maximum prevalence of infection in the immunized group was 60% (Fig. 5-4B). Correspondingly, the mean parasitemia of immunized fish was consistently lower than control fish (Fig. 5-4C). When comparing the mean parasitemia of these two groups, the greatest difference of 2.5 common logarithms was observed 7 dpi. However, the difference between these means was not significant.

The variability in the antibody response of individual fish in the same treatment group was immense (Fig. 5-4E). Although there were no significant differences between the response of immunized and control fish, the mean ELISA value of samples from immunized fish increased as much as ~35% over that observed on 0 dpi, whereas the control group had a maximum increase of ~20%. Moreover, a weak correlation coefficient of 0.34 and 0.25 was drawn between parasitemia and the antibody responses of immunized and control fish, respectively (Table 5-2).

Intraperitoneal administration (FCA). Immunization with an emulsion of ES products and FCA conferred marked protection against *T. danilewskyi* challenge. Although the prevalence of infection and the mean parasitemia was substantially greater in the control group, no mortality was observed (Figs. 5-5A, 5-5B, and 5-5C). From 7 to 28 dpi, all control fish were infected with *T. danilewskyi* (Fig. 5-5B). In comparison, the prevalence of infection in the immunized group reached a maximum of 80% on 28 dpi. By 56 dpi, the prevalence of infection in the immunized and control group was 0 and 60%, respectively. Those fish, immunized with an emulsion of ES products and FCA, and subsequently challenged with *T. danilewskyi* had ~60% fewer parasites than those inoculated with control solution (Fig. 5-5C). Compared to the parasitemia observed in the control group, fish immunized with ES products and FCA had a longer lag period with the maximum number of parasites occurring on 28 dpi. The greatest difference between the two groups was observed on 7 dpi, where the mean parasitemia was nearly five common logarithms lower ($P < 0.008$) than that observed in the control group. Between day 28 and 56, a marked decline in parasitemia was observed in both groups. However, no parasites were found in the blood of the experimental fish on day 56.

The mean ELISA values of fish antisera were not significantly affected by immunization or *T. danilewskyi* infection (Fig. 5-5E). Nevertheless, for each observation day, the mean value of immunized fish was two- to six-fold lower than the control group. Although 2.5-fold lower than that of the control group, the peak antibody response was observed 2 wk earlier. Further, the percent of control value confirms that the response of fish administered ES products with FCA intraperitoneally was less than or equal to that of fish inoculated with control solution (Table 5-1). Notwithstanding, a correlation coefficient of 0.46 ($P < 0.05$) was drawn between the abundance of parasites in immunized fish and the humoral immune response (Table 5-2).

Approximately 4 mo later, an additional experiment was designed to: 1) confirm that immunizing with ES products, emulsified in FCA, conferred protection against *T. danilewskyi*; and 2) determine whether the magnitude of protection was related to the concentration of ES products (10X, 5X, or 1X) in the initial inoculum. Unfortunately, the Biological Sciences Aquatic Facility became contaminated with *Aeromonas salmonicida* , which necessitated the termination of this experiment. Three subsequent attempts were made to conduct this experiment. Despite rigorous efforts (i.e., though cleaning of the environment, minimizing possible modes of transmission, and prophylactic-treatment of fish) to eliminate this problem, the experimental fish exhibited clinical signs that typified bacterial infection. From these occurrences, I concluded that the naïve fish bear a subclinical infection that manifests itself with concomitant antigenic challenge. Inasmuch as little support was obtained from the Biosciences Animal Services, no further effort was made to repeat this experiment.

Gel electrophoresis/immunoblotting. Gel electrophoresis and immunoblotting was used to visualize the antigenic profile of ES products. In an introductory experiment, nitrocellulose membranes were reacted with immune (50 dpi) or normal fish serum. Excretory-secretory molecules of molecular weight 104 and 72, and 59 (doublet) kDa (unpublished data, D.A. Plouffe) were recognized by immune serum (Fig. 5-6). Serum collected from uninfected animals only recognized the 59 kDa molecule. At similar antibody concentrations, no bands were observed on membranes containing control solution. Likewise, no bands were observed on negative control lanes (with ES products or control solution) in which the fish serum, anti-carp IgM hybridoma supernatant, or the enzyme-labeled antibodies were omitted.

Sodium perchlorate treatment

Intravenous administration. Data obtained from fish immunized intravenously with sodium perchlorate-treated (i.e., whole dead) trypanosomes indicated that this treatment had a partial protective effect. While no mortality was observed in the immunized fish, 40% of the control fish died by 28 dpi (Table 5-3). During the first 2 wk of the infection, the immunized group had a lower prevalence of infection, 60 and 80%, respectively, than that observed in the control group. However, by 28 dpi, *T. danilewskyi* was found in the blood of all fish (Table 5-4). Although not significantly different from the control group, intravenous immunization with sodium perchlorate-treated trypanosomes reduced the mean number of parasites by ~25%. The most notable difference between the two treatment groups (16-fold) was observed when the control fish reached peak parasitemia at 14 dpi (Table 5-5). No significant correlation was drawn between parasitemia and the antibody response of fish inoculated intravenously with sodium perchlorate-treated parasites or control solution (Table 5-6).

Intravenous immunization with sodium perchlorate-treated trypanosomes did not significantly affect the humoral immune response, as mean ELISA values of samples collected from immunized fish was like that observed in the control group on 0 dpi (Table 5-7). Further, the secondary immune response of infected immunized fish was never significantly different from that observed with samples collected from control fish. Although these comparisons were not statistically different, the mean ELISA value (28 and 56 dpi) of the immunized group was at least five-fold greater than that of the control group. In addition to demonstrating the increase in ELISA values on these days, the percent of control values suggested a suppression or depletion of free antibodies during the first 2 wk of the infection (Table 5-8). The percent of control value on 7 dpi is not presented, as only one sample was available for this analysis.

Because intravenous inoculation may not have been successful in all fish, the infection dynamics of individuals was examined in conjunction with the ELISA values. Of all fish, the profile of a single immunized fish was markedly different. Notwithstanding, it may be erroneous to conclude that this fish was the only one that received a successful inoculation as: 1) mortality (40%) occurred only in the control group; and 2) early in the infection, four of five control fish had >1 X 10^6 parasites ml^{-1} blood. In contrast, only one of five experimental fish had parasitemia of this magnitude. Also, the decreased antibody response in the aforementioned immunized fish was also observed in other fish with moderate to high parasitemias.

Regardless of the inoculum route, the red cell volumes of fish (injected with sodium perchlorate-treated trypanosomes or control solution) followed similar trends. Hence, data relevant to this experiment will be presented in this section. Comparisons between mean red cell volumes of immunized and control fish were not significant (Table 5-9). The only exception to this was observed in the group inoculated intraperitoneally with FCA. In this group, the mean value (14, 28, and 56 dpi) of immunized fish was nearly 7% greater ($P < 0.03$) than that of the control group. Over the time course, intraperitoneal inoculation (without adjuvant or with FCA) and *T. danilewskyi* infection did not affect the mean red cell volume significantly. In contrast, the remaining groups had a significant increase between 28 and 56 dpi. Comparisons of red cell volumes between the non-immunized groups were not done. Notwithstanding, a ~12% decrease in the red cell volume of the non-immunized infected group was observed between the first and the fourth week of the infection.

Intramuscular administration. Intramuscular immunization with trypanosomes killed by sodium perchlorate had no protective effect as: 1) greater mortality occurred in the immunized group than in the control group (Table 5-3); 2) all fish in the control and immunized group were infected 7 days after challenge with *T. danilewskyi* (Table 5-4); and 3) parasitemia of immunized and control fish were comparable (Table 5-5). As indicated by the prevalence and the course of infection, control fish were able to eliminate the trypanosomes from the blood slightly quicker than immunized fish. No strong linear correlation could be drawn between the humoral response and the abundance of parasites in fish given intramuscular inoculations (Table 5-6).

Intramuscular immunization or *T. danilewskyi* infection, or both did not significantly affect the mean ELISA values (Table 5-7). For both groups, antibody recognition of trypanosome antigens peaked at 14 dpi and decreased to values less than or equal to that observed 0 dpi. Relative to the mean ELISA values observed on 0 dpi, the increase of antibody in the control group was greater than that in the immunized group (7 and 14 dpi) (Table 5-8). However, on 28 dpi, the percent of control value approached 150%.

Intraperitoneal administration (no adjuvant). Although higher parasitemia and prevalence of infection was observed in fish immunized intraperitoneally with sodium perchlorate-treated parasites, no mortality was observed (Table 5-3 to 5-5). In the control group, two of the five fish died with high parasitemia ($> 2 \times 10^7$ ml^{-1} blood). Over the first 7 days of the infection, no significant differences were present when parasitemia of immunized and control fish were compared. However, as the infection progressed (14 to

56 dpi), the mean number of parasites ml^{-1} blood in immunized fish was consistently higher ($P < 0.03$) than that in control fish (Table 5-5). A weak positive correlation was seen between the number of parasite ml^{-1} blood and the detection of antibodies in intraperitoneally immunized and control fish (0.41 and 0.37, respectively) (Table 5-6).

Prior to *T. danilewskyi* infection, the mean ELISA value of the immunized group was four-fold greater ($P < 0.05$) than that recorded for the control group (Table 5-7). Although the ELISA values of immunized fish were consistently higher for the remainder of the experiment, these comparisons were not significant. As observed with the other inoculation routes, the maximum mean ELISA value was observed 14 dpi. After the second week of the infection, the ELISA values of each group declined approximately two-fold. The percent of control values suggested that the humoral immune response of immunized infected fish was less than or roughly equivalent to that observed in the control fish (Table 5-8).

Intraperitoneal administration (Freund's adjuvant). Adjuvant (FIA or FCA) given with sodium perchlorate-treated trypanosomes affected the outcome of the disease modestly. For each of these immunization groups, mortality was at least 20% less than that of the respective control group (Table 5-3). Overall, the prevalence of infection in fish, immunized with treated trypanosomes suspended in adjuvant, was similar to that of the control group (Table 5-4). Freund's complete adjuvant given with sodium perchlorate-treated *T. danilewskyi* did not significantly affect parasite numbers within the blood (Fig. 5-5C). However, it appears that partial protection was conferred in the immunized group given FIA, as the mean parasitemia on 7 dpi was 30% less ($P < 0.04$) than that in the respective control group (Table 5-5).

With greater than a two-fold increase in the mean ELISA value (0 dpi), immunization with sodium perchlorate-treated trypanosomes suspended in FIA significantly enhanced ($P < 0.05$) the antibody response (Table 5-7). During the first 2 wk of the infection, the mean ELISA values of the immunized group remained approximately two-fold higher than that observed in the control group. Thereafter, the ELISA values of experimental and control fish were similar. A strong positive correlation ($P < 0.0001$) was drawn between the abundance of parasites and the antibody response of fish treated with whole dead trypanosomes suspended in FIA (Table 5-6). In contrast an insignificant weak correlation was observed in the respective control group.

In the fish given inoculations with FIA, an initial increase of antibody production was suggested by percent of control values recorded on 7 dpi (Table 5-8). Thereafter, the percent of control value decreased drastically to values below 5%. Examination of data of individual fish revealed that one control fish with over a 10,000-fold increase in the ELISA value strongly influenced this analysis. Removal of data collected form this fish resulted in values that have been previously observed. Namely, a maximal value of 170% was observed 14 dpi. Thereafter, the percent of control values declined to approximately 120% and 70% on 28 and 56 dpi, respectively. With regard to statistical comparisons of mean ELISA values, omission of the data collected from this fish did not affect the outcome of the analyses.

Neither immunization (whole dead trypanosomes suspended in FCA) or *T. danilewskyi* infection affected the antibody response of experimental fish significantly (Table 5-7). In general, the mean antibody response of immunized fish followed a similar pattern seen in the control group. The most notable difference was the maximum ELISA value observed on 14 dpi that was preceded by a slight decrease in antibodies. Nevertheless, relative to that observed on 0 dpi, this increase was similar to the response of control fish (Table 5-8). As demonstrated with the mean ELISA values, the percent of control value indicated that the peak antibody response was followed by a marked decline in parasite specific antibodies. Of the two groups administered FCA, a moderate positive linear correlation ($P < 0.006$) between parasite number and the antibody response was only observed in the control group (Table 5-6). In this group, all the samples collected from fish with > 4 X 10^7 parasites ml^{-1} blood had ELISA values with at least a four-fold increase over that observed on 0 dpi.

Infection dynamics of non-immunized control fish

As referred to in the experimental design, each immunization trial (i.e., ES products, whole dead parasites, trypanosome lysate, water-soluble molecules or detergent-soluble molecules) included two groups of fish that served as positive and negative controls (i.e., non-immunized infected and non-immunized uninfected). Results obtained from these two control groups are presented below.

Inasmuch as the trypanosome lysate and ES product immunization experiments were conducted simultaneously, data collected from the non-immunized groups were applied in the analyses of both of these experiments. Consequently, the infection dynamics data will be presented once. Except for one heavily infected fish, all non-immunized fish included in the experiments (ES product and trypanosome lysate) survived (Fig. 5-7A and 5-8A). The prevalence of infection in the non-immunized infected group reached 100% by 28 dpi (Fig. 5-7B and 5-8B). All fish in this group remained infected until the experiment was terminated. During the first week, the course of infection in the non-immunized infected group was like that observed in fish inoculated with parasite extract or control medium (Fig. 5-1C to 5-5C and B-1C to B-5C). However, after the second week of infection, the mean parasitemia in the non-immunized infected group was consistently higher ($P < 0.05$) than that of the other cohort groups.

Of the non-immunized infected fish included in the sodium perchlorate-treated trypanosome immunization experiment, only one fish with massive parasitemia died (Table 5-3). Much like that observed in other groups, all fish were infected with *T. danilewskyi* by 7 dpi (Table 5-4). By 56 dpi, only one of the four surviving fish had cleared the infection from the blood. With one exception, the course of infection in these fish was not statistically different from that of fish immunized with whole dead trypanosomes or control solution (Table 5-5). The only significant difference ($P < 0.003$)

from that in the non-immunized infected group was observed in fish given an intraperitoneal inoculation of control medium (no adjuvant). In the latter group, the mean abundance of parasites was reduced by approximately three common logarithms (Table 5-5).

None of the non-immunized fish, included in the water-soluble extract immunization experiment died (Fig. 5-9A). One of the six fish in the non-immunized infected group never developed a patent infection. Notwithstanding, the prevalence of infection was unusually low (Fig. 5-9B). This was due to two very lightly infected fish in which parasites were detected intermittently. Nevertheless, the course of infection was not significantly different from that observed in infected immunized or control fish (Fig. B-6C to B-9C).

No death occurred in any of the non-immunized fish included in the detergent-soluble extract immunization experiment (Fig. 5-10A). While the non-immunized uninfected group remained uninfected, the prevalence of infection in the non-immunized infected group reached 100% by 7 dpi (Fig. 5-10B). The course of infection in these fish was like that of all groups given intravenous or intramuscular inoculations of detergent-soluble extracts or control solution (Fig. B-10 to B-14). In comparison, intraperitoneal administration of FIA or FCA suspended in control solution significantly reduced ($P <$ 0.04) the mean parasitemia.

Relative antibody response of non-immunized control fish

The presence of anti-*T. danilewskyi* antibodies in non-immunized fish (infected or uninfected) was detected with the ELISA, using various antigen preparations. Namely:

ES products. On 0 dpi, the mean ELISA values of the non-immunized groups were very similar (Fig. 5-7E). The mean antibody response of the infected and uninfected group did not differ significantly over the course of the infection. Nonetheless, the infected group had nearly a two-fold increase in the ELISA values (0 vs 56 dpi). The mean value of the infected group was strongly influenced by data collected from a single fish. Sera collected from this fish resulted in optical densities that were three to four times greater than the mean ELISA value observed on 0 dpi. During the course of infection, the increase in the ELISA values observed in the non-immunized infected group did not correlate significantly with the number of trypanosomes ml^{-1} blood (Table 5-2). Blood from all the non-immunized/uninfected fish remained negative for *T. danilewskyi*.

Sodium perchlorate-killed trypanosomes. Relative to that observed on 0 dpi, recognition of sodium perchlorate-treated trypanosomes by antiserum collected from infected fish increased by an insignificant amount (1.5-fold) (Table 5-7). Between 14 and 56 dpi, the ELISA values decreased by more than three-fold ($P < 0.05$). The parasitemia of the non-immunized infected fish did not correlate significantly with the presence of antibodies (Table 5-6). Inasmuch as a contaminated syringe was inadvertently used on fish in the non-immunized uninfected group, these fish were omitted from the experiment. Moreover, because the acclimation period could not be reproduced in a timely manner, no effort was made to replace the fish.

Trypanosome lysate. On 0 dpi, the mean antibody response of the non-immunized groups to trypanosome lysate antigens was very similar (Fig 5-8E). When comparing infected to uninfected groups, *T. danilewskyi* infection did not significantly affect the ELISA values. Nonetheless, the infected group had a three-fold increase in the mean ELISA values between 0 and 56 dpi. Some of the variability in the infected cohort can be attributed to two of the five fish. Curiously, during the infection the detection of parasite specific antibodies in these two fish dropped below that observed on 0 dpi. Notwithstanding, the percent of control values increased approximately three- and six-fold by 28 and 56 dpi, respectively (Table B-1). A significant linear correlation was not found between the abundance of parasites and the antibody response of the non-immunized infected fish (Table B-2).

Water-soluble extracts. The mean ELISA values of the non-immunized infected and the non-immunized uninfected group was identical on 0 dpi. (Fig. 5-9E). During the first week of the infection, the differences between the two non-immunized groups were not statistically different. However, by the second week the antibody response of infected fish was nearly two-fold ($P < 0.05$) greater than that of the uninfected group. Comparisons of ELISA values between the two groups were not significantly different on 28 and 56 dpi. Moreover, the ELISA values observed on 28 and 56 dpi were not significantly different from that observed on 0 dpi. With a correlation coefficient of 0.37, the number of parasites ml^{-1} blood in the non-immunized infected group correlated weakly ($P < 0.05$) with the increase in ELISA values (Table B-4). For the duration of the experiment, the non-immunized uninfected fish remained uninfected with *T. danilewskyi*.

Antigen-induced proliferation of leukocytes

Immunological memory was present in PBL obtained from recovered fish, as blastogenic responses were observed when immune leukocytes were treated with 10 or 100 μg of parasite protein. The optimal concentration of PBL was 10^6 cells per well, as the mean optical density of wells containing 10^6 cells was significantly greater ($P < 0.0001$) than those containing no or 10^5 cells (Fig. 5-11). Although significant differences ($P < 0.04$) were observed between immune and nonimmune cells (10^6/well) stimulated with 10 or 100 μg of protein, greatest differences (20%) were observed when 10 μg of protein was used to activate the leukocytes.

Passive transfer of serum

All fish that were inoculated with serum (immune or nonimmune) and challenged with *T. danilewskyi* were confirmed to be infected 7 dpi. However, fish immunized with immune serum had significantly lower numbers ($P < 0.02$) of parasites ml^{-1} of blood than those fish that received no immunization or nonimmune serum (Fig. 5-12). This difference in parasitemia (~ 25%) was observed 7, 14, and 21 dpi. The course of infection in fish inoculated with nonimmune serum was not significantly different from those that were not immunized.

DISCUSSION

The single administration of various antigen preparations via different vehicles and routes was studied. Clearly, the immune responsiveness of fish was affected by numerous other factors such as animal age, temperature, dosage, time intervals, and number of booster injections. Because it was not feasible to examine all parameters simultaneously, some variables were held constant. For example, the fish were consistently maintained at 20°C, as this temperature has been reported to be favorable for the induction of an immune response [52, 228, 229]. Inasmuch as resistance to reinfection appeared to be independent of the intensity of the infection (see Chapter III), the administration dose was kept constant. Parenthetically, this dose represented the median parasitemia at the peak of a primary infection. I have not included the age of the fish, as this would have been a subjective assessment. Rather, all active immunization experiments were conducted with fish 9.5 to 11 cm in standard length.

The results obtained from the immunization experiments are summarized in Tables 5-10 to 5-14. As demonstrated in these tables, the response of fish to immunization comprises three main groups.

1) Immunization with parasite extract conferred significant levels of protection. In this single case, the decreased prevalence of infection and parasitemia coincided with a moderate, but insignificant, suppression of the antibody response.

2) Insignificant levels of protection. The infection dynamics of immunized fish was similar to that observed in fish inoculated with control medium. Contingent on the antigen preparation and inoculum route, the lack of protection coincided with an increase, decrease, or no difference in the detection of parasite-specific antibodies.

3) Immunization enhanced susceptibility to *T. danilewskyi* infection. In these instances, the presence of antibodies directed against trypanosomes was similar to that observed in control fish given a primary infection.

Rather than presenting a detailed discussion of each immunization strategy, I intend to offer a presentation of concepts that were associated with these categories. Additionally, the discussion will include: 1) information relative to the association between red cell volume and anemia; and 2) a brief discussion of the administration routes.

Immunization conferred protection against *T. danilewskyi.*

Of this entire survey, the most promising observation was that of ES products administered intraperitoneally with FCA (see summary Table 5-10). These findings were unique inasmuch as the protective immunization with ES products have not been described in fish kinetoplastids. The effectiveness of this immunization was most apparent during the first week of the trypanosome infection. At 7 dpi, the mean parasitemia was nearly five common logarithms lower than that observed in the control group. After the second week of the infection, the parasites attained numbers that were comparable to that in control fish. Collectively, these observations suggested that greater efficacy could be achieved by administering a secondary (booster) immunization. Further, valuable information could be obtained from therapeutic administration of ES products and FCA.

Aside from the direct effect of antibodies and their effector mechanisms, it was likely that cell-mediated immune factors were associated with the clearance of the infection. My experiment with ES products and FCA supported this hypothesis. While nonspecific factors may have functioned as a defense mechanism, the requirement that FCA be given with ES products was interpreted to suggest that this administration facilitated the development of a specific cell-mediated response to the parasite. As presented in the literature review (Chapter I), our knowledge of the cell-mediated immune responses is limited to leukocyte infiltration in histological sections [217, 249], delayed-type hypersensitivity reactions [92, 248, 271], macrophage migration inhibition assays [248, 271], and adoptive transfer of leukocytes from immune to naïve fish [148]. Interestingly, no information is available on the role of cell-mediated immunity in fish trypanosome infections. A pilot study I conducted suggested that immunological memory was present in peripheral blood lymphocytes obtained from recovered fish (112 dpi), as blastogenic responses were observed when immune leukocytes were treated with 10 or 100 μg of parasite protein. With this in mind, useful data may emerge from similar experiments that assess the blastogenic response of defined lymphocyte populations.

As discussed previously, a positive correlation between parasitemia and ELISA values was observed in the group immunized intraperitoneally with ES products suspended in FCA. Inasmuch as the resistance of this group was markedly enhanced by immunization, it seems reasonable to speculate that those fish that mounted a T_{H2}-like immune response (production of factors that have B-cell stimulation activities) were more susceptible. Conversely, those that elicited a T_{H1}-like response (production of factors that induce delayed-type hypersensitivity and generation of cytotoxic lymphocytes) were rendered more resistant. Needless to say, this supposition would need to be substantiated by future experimentation.

Insignificant levels of protection

With no apparent effect on parasitemia, intramuscular administration of soluble antigens caused a significant reduction in the detection of parasite-specific antibodies (see summary Table 5-13). Certainly, this observation may be indicative of a number of immunological processes. For instance, upon encountering the parasite antigen, it was possible that the immune response entered a state of unresponsiveness called tolerance. In other fish, induction of suppression resulted from administration of protein antigen (i.e., bovine serum albumin, chicken gamma globulin, *A. salmonicida*) or hapten-carrier antigens [157, 158, 235, 245]. Moreover, the earliest of these studies illustrated that the suppression was dependent on the route of administration and the physiochemical nature of the immunogen [245]. Secondly, investigations of other protozoan systems attributed the suppressive effect to the presence of parasite molecules. Specifically, a soluble molecule of *Glugea* spp. stimulates the host leukocytes to release mediators (i.e., prostaglandin) that suppressed the initiation of the humoral response. In another blood-borne protozoan system, workers have ascribed parasite-induced immunosuppression to the presence of competing antigen that regulates the response to an unrelated antigen (i.e., antigenic competition) [152, 289]. Conceivably, this could have occurred in my experiment by overwhelming the immune system with irrelevant parasite protein present in the unfractionated extract. Thirdly, in an adoptive transfer study, the presence of T-suppressor-like cells were suggested to diminish protective mechanisms against *C. salmositica* [92]. Conceptually, these cells could inhibit the humoral immune response by: 1) suppressing lymphocytes bearing specific idiotypic determinants related to antigen recognition; and 2) removing the stimulus before an immune response could be generated by suppressing active antigen presenting cells [107]. While these latter studies offer interesting possibilities, it was unlikely that these phenomena provided a reasonable explanation for results observed in my experiments, unless they were associated with a specific administration route. Lastly, it was possible that the decreased detection of antibodies was associated with depletion. That is to say, the parasite-specific antibodies were trapped in antigen-antibody complexes and were consequently undetectable by the ELISA. To address this concern, future experiments using direct immunofluorescent staining (i.e., fluorescein isothiocyanate-labeled anti-fish IgM) and flow cytometry could determine whether antibodies were bound to the surface of trypanosomes that were freshly isolated from blood. Alternatively, immune complexes may require dissociation (i.e., lowering pH) before performing an ELISA.

In the majority of the trials, immunized fish had a mean abundance of parasites and antibody responses that were not significantly different from the respective control groups (Table 5-10, 5-12, and 5-13). As pointed out several times in the result section, a marked difference in the mean response was observed. However, the responses of individual fish within a cohort varied exceedingly. Notwithstanding, relative antibody responses seen in my experiments were not unlike those previously observed in carp infected with *T. danilewskyi*. For instance, at optimal antibody concentrations, the relative antibody responses of individual immune fish ranged between 10 and 80% [215]. Numerous factors (i.e., genetic variation, nutritional status, and cross-reactive epitopes) contributing to the heterogeneous responses of fish have been identified in the literature. For example, it was important to acknowledge that experimental fish may have had immunity from previous parasitic infections with cross-reactive epitopes. More specifically, several reports have shown that cross-protection against *I. multifiliis* was conferred by immunization with another ciliate [71, 109, 110, 295], or an ectoparasitic platyhelminth [50]. Likewise, immunization with *I. multifiliis* or *T. pyriformis* protected goldfish against other ectoparasitic protozoa [181]. While this concern remained a possibility in my studies, it seemed unlikely, inasmuch as; 1) all fish were confirmed to be uninfected with haemoflagellates when the experiments began; and 2) upon gross examination, metazoan parasites were never detected in any of the fish dissected in our laboratory. Additionally, of all the non-immunized infected fish included in this chapter, only two fish had parasitemias (< 1000 trypanosomes ml^{-1} blood) that were comparable to that observed in recovered fish given a secondary infection (see Chapter III). However, the ELISA values of samples collected from these two fish did not differ significantly from that observed in susceptible fish with > 1000 parasites ml^{-1} blood.

Nutritional status and the social behavior of fish may account for some of the heterogeneity observed between cohorts. Barrow [27, 28] reported that the production of antibodies was associated with these factors. At present, it is unclear if the production of antibodies was responsible for resolving *T. danilewskyi* infections because there was no relationship between protection and anti-parasite antibody. Further, two publications have demonstrated that anorexia and the resulting decrease in plasma protein reduced the severity of haemoflagellate infections [138, 176]. Notwithstanding, the role of other nutritional components in disease resistance should not be underestimated, as survival rates were influenced by vitamins [281, 282].

In *Cryptobia* and *Trypanoplasma* host-parasite systems, a few workers have reported those genetically transmitted factors of carp, salmon, and brook charr contribute to host resistance. This resistance varies within a species and between species. [43, 101, 291, 292]. Genetically, carp can be divided into high and low antibody responders. Those that were able to generate a high antibody response were less susceptible to *T. borreli*. Correspondingly, highly susceptible carp responded with low antibody production to parasite antigen or dinitrophenyl-keyhole limpet haemocyanin [291, 292]. Using the *in vitro* plasma incubation test as an immunoassay, another study determined that innate resistance (i.e, complement-mediated cytolysis) in brook charr was determined by a single Mendelian locus [101]. Although genetically related resistance has not been substantiated for *T. danilewskyi* infection in goldfish, a supposition based on this information would provide an additional explanation for the heterogeneous responses within a treatment group. Further, it may account for the relatively low antibody responses that were observed in the immunized or control fish that were susceptible to *T. danilewskyi*.

Aside from the heterogeneity observed between cohorts, it was surprising that the secondary response (ELISA values or blastogenesis) increased by modest amounts. While countless immunological processes may be responsible for the magnitude of the memory response, it is only possible for me to offer a few likely explanations. In fish, the proliferative capacities (clone size) of memory precursor lymphocytes were fairly small and were comparable to that observed in cells from a naïve donor. In comparison, the clone sizes of naïve mammalian cells were at least five times larger. Further, the memory precursors from rats had 25-fold larger clone sizes than that observed in trout (reviewed by [153]). Also, the binding strength between the receptor on the antibody and the epitopes on an antigen (affinity) and lack of affinity maturation may be closely associated with the seemingly low responsiveness of the immunized fish. In ectothermic species (i.e., trout, salmon, giant grouper, *Epinephelus* sp., snapper, *Lutjanus* sp., and horned shark (Order Heterodontiformes)), the increase in the average affinity of antibodies produced during secondary immune responses to antigen appears to be uncommon or weakly expressed, as they do not possess the necessary genetic elements (i.e., isotype switching or somatic mutation) [153].

In my mind, the function of antibodies in a *T. danilewskyi* infection remains an enigma. On the one hand, passive transfer of immune plasma [298], serum, or purified IgM [215] was successful in conferring protection. Neutralization of infectivity has been proposed as an effector mechanism, as *T. danilewskyi* incubated at 20°C for 2 hr in immune plasma were not infective to goldfish [298]. While the authors of these studies attributed the resolution of the infection to the production of trypanosome-specific antibodies, one report simultaneously illustrated that large numbers of trypanosomes are present in carp 2 to 3 wk after the detection of the peak antibody response [215]. Although a significant increase in antibody was only observed in one instance (see summary Table 5-13 and Appendix B), my results repeatedly showed a positive correlation between parasitemia and the induction of an antibody response. Taken together, I would argue that the efficacy of antibodies was contingent on the phase of the infection. For example, passive transfer of antibodies was successful because the antibody was present as the inoculated trypanosomes were beginning to establish an infection. In contrast to the course of a primary infection, the parasites have established a peak infection during the lag phase of the humoral response. By this time, the antibodies were insufficient to control the infection. It was possible that the ineffectiveness in controlling trypanosome infections was associated with the seemingly low antibody concentration. Alternatively, it was possible that other mechanisms controlled the resolution of the infection.

It appears as though antibodies do not adversely affect the multiplication or metabolism of *T. danilewskyi*, as *in vitro* cultivation in immune sera supported the parasites as well as sera from naïve fish (see Chapter 4). Ironically, one study on *T. brucei* demonstrated that anti-trypanosome antibodies stimulated parasite proliferation both *in vivo* and *in vitro* [246].

Immunization enhanced susceptibility to *T. danilewskyi*

In five of the immunization trials, the susceptibility of goldfish to *T. danilewskyi* was significantly greater than that of the respective control group (see summary Table 5-10 and Appendix B). In all of these cases, the antibody response was not significantly different from that observed in the controls (see summary Table 5-13 and Appendix B). As observed with *T. cruzi* [202], *Leishmania* spp. [70, 137], *Entamoeba histolytica* [237, 286, 287], and *Toxoplasma gondii* [226], it is conceivable that *T. danilewskyi* suppressed host responses (other than humoral) that rendered the vertebrate more susceptible to disease. Mechanisms of suppression by the mentioned parasites include: 1) reduced mitogen-induced lymphocyte proliferation; 2) inhibited the induction of interferon-gamma induced surface Ia antigen expression; 3) suppressed induction of interleukin-two receptor following mitogen stimulation; 4) down-regulated macrophage effector and accessory cell potential; and 5) suppressed macrophage expression of major-histocompatibility antigens (class I and II).

An additional explanation for enhanced susceptibility may involve the parasite's ability to influence the host's physiology and the production of growth factors. As presented in Chapter I, parasites can secrete molecules (cytokine-like and hormones) that allow exacerbation of parasite numbers. Alternatively, the parasite secretes factors that alter the host response.

The apparent increase in susceptibility of immunized fish may also be explained by the fact that administration of control medium conferred significant levels of protection. This hypothesis was supported by the observation that fish, given control solution (with or without adjuvant), had significantly lower numbers of parasites than that observed in the non-immunized infected fish (see summary Table 5-11 and Appendix B). In the absence of adjuvant, it was possible that components (vitamins, amino acids, etc.) present in the control media enhanced the physiological well being of the fish. As observed in other studies [114, 210], it was possible that administration of adjuvants elicited a nonspecific immune response, which served an important defensive function. In general, adjuvants were used to boost the immune response of fish when an antigen has low immunogenicity or was only available in small quantities [114, 134, 136, 199, 210, 221]. Freund's incomplete adjuvant is a formulation of paraffin oil and an emulsifying agent that disperses the oil into small droplets surrounding the antigen. In addition to that contained in FIA, FCA included killed *Mycobacterium butyricum*. A muramyl component of the mycobacterial cell wall can activate macrophages to increase the production of reactive oxygen metabolites and consequently enhance nonspecific resistance to infection. Additionally, the increased secretion of cytokines (i.e., interleukin-1, and colony-stimulating factor) from activated macrophages may result in the activation of T-cells and the differentiation of B-cells (reviewed by [2, 11]).

Red cell volume/anemia

Trypanosoma danilewskyi can cause severe anemia in goldfish at or following peak parasitemias [81, 139, 230, 283]. Anemia is also a common pathologic feature of other fish trypanosome infections [154, 185]. The anemic condition is often characterized by watery blood, pale-colored gills [230], and a significant decline of the packed red cell volume [139, 283]. Although a few differences between the mean red cell volume of immunized and control fish were detected, no consistent trends (relative to the antigen preparation, administration route, or vehicle) were evident (see summary Table 5-14). For this reason, results from the non-immunized infected fish (included in the five immunization studies) were used to represent the association between the red cell volume and parasite abundance.

During the first 2 wk of the infection, the mean red cell volume of non-immunized infected fish declined by nearly 10%. This decrease was associated with the magnitude of parasitemia, as a significant correlation ($P < 0.0001$) was drawn between the red cell volume and the number of parasites. On 14 and 28 dpi, the mean parasite abundance of > 2×10^7 trypanosomes ml^{-1} blood was associated with a haematocrit of 31 to 33%. After the acute phase of the infection, the red cell volume recovered to values similar to that observed on 0 dpi (39 to 40%).

As mentioned previously (Chapter I), *T. danilewskyi* infection causes severe histopathological changes in the haematopoietic organs and excessive disintegration of red blood cells [81]. Accordingly, these authors attributed anemia to the malfunctioning spleen and inadequate haematopoietic capacity of the kidney. Islam and Woo (1991) elucidated two additional factors that cause anemia. Using *in vitro* techniques, they identified that ES products of live trypanosomes (8×10^6 ml^{-1}) contain a haemolysin that can lyse 100% of the red blood cells (224 per microscopic field) in 6 hr. The second factor, haemodilution, correlated with the number of parasites present in the blood.

It appears that experimental manipulations may contribute to the decreased red cell volume, as significant differences were observed over time in the non-immunized uninfected group (included in the detergent-solubilized and water-solubilized experiments). For example, my experiments were designed and analyzed as repeated measure observations. Under these conditions, it was possible that the small amounts of blood withdrawn (~60 µl) affected the red cell volumes. Also, the influence of the anesthetic must be considered. In fish, side effects of tricaine methane sulfonate include persistent leaching from the muscle that may affect blood chemistry and reduce branchial movement. This in turn leads to hypoxia (i.e., reduction of oxygen supply to the tissues), which can cause acidification of the blood and an increase in red blood cell size [96]. Studies on other poikilotherms have reported that exposure to tricaine methane sulfonate affects glucose concentrations in the blood (i.e., hyperglycemia) [276], and salt metabolism [198].

Administration route

In mammals, the route of administration determines which organs and cell populations will be involved in immunity. Differences in the proportion of lymphoid cells, populating these organs, generate differences in the quality of the secondary immune response [163]. However, the system of lymphoid organs in fish is less sophisticated than in mammals (reviewed by [40, 58, 153]). For example, the thymus is reported to be the origin of all fish lymphocytes. Further, 65 to 68% of the fish thymus lymphocytes have surface Igs, which are regarded as a characteristic of mammalian B-cells. Inasmuch as fish lack bone marrow and lymph nodes, the pronephros of the kidney serves the function of both. For these reasons, one could expect that inoculation route affects the immunological system of the fish differently than in mammals. A review of immunization trials against bacterial species demonstrated important variations on the species of fish as well as the inoculum route [33]. As a result, it was difficult to come to a consensus about the route of administration that was most efficacious.

From my studies, it becomes evident that the combination of antigen preparation and vehicle were the discriminating factors. Of the three groups immunized intraperitoneally with ES products, significant levels of protection were only observed in the groups given this antigen suspended in FCA. Further, intraperitoneal administration of FCA with the other parasite extracts did not render the fish resistant to *T. danilewskyi*.

Table 5-1. Percent of control value[a] of the antibody response of fish immunized with excretory-secretory products of *Trypanosoma danilewskyi.*

	TIME (days postinfection)			
Administration route	7	14	28	56
Intravenous	110	133	157	99
Intramuscular	90	78	56	85
Intraperitoneal	90	91	68	77
Intraperitoneal/FIA[b]	92	101	112	92
Intraperitoneal/FCA[c]	89	101	94	81

[a] ELISA values transformed into the percent increase or decrease of that observed 0 dpi, which equals 100%

[b] Freund's incomplete adjuvant

[c] Freund's complete adjuvant

Table 5-2. Correlation coefficient (r) between parasitemia and the antibody response of fish immunized with excretory-secretory products of *Trypanosoma danilewskyi.*

Treatment	Administration route	r	*P*-value
ES product	Intravenous	0.39	N.S.[a]
Control solution	Intravenous	0.11	N.S.
ES product	Intramuscular	0.27	N.S.
Control solution	Intramuscular	0.70	$P < 0.008$
ES product	Intraperitoneal	0.57	$P < 0.02$
Control solution	Intraperitoneal	0.32	N.S.
ES product/FIA[b]	Intraperitoneal	0.34	N.S.
Control solution/FIA	Intraperitoneal	0.25	N.S.
ES product/FCA[c]	Intraperitoneal	0.46	$P < 0.05$
Control solution/FCA	Intraperitoneal	0.33	N.S.
Non-immunized infected	N/A[d]	0.06	N.S.

[a] not significant
[b] Freund's incomplete adjuvant
[c] Freund's complete adjuvant
[d] not applicable

Table 5-3. Survival (%) of fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi.*

		TIME (days postinfection)				
Treatment	Inoculum route	0	7	14	28	56
Whole dead	Intravenous	100	100	100	100	100
Control solution	Intravenous	100	100	100	60	60
Whole dead	Intramuscular	100	100	60	60	60
Control solution	Intramuscular	100	100	100	100	100
Whole dead	Intraperitoneal	100	100	100	100	100
Control solution	Intraperitoneal	100	80	60	60	60
Whole dead/FIA[a]	Intraperitoneal	100	100	100	80	80
Control solution/FIA[b]	Intraperitoneal	100	100	60	60	60
Whole dead/FCA	Intraperitoneal	100	100	100	100	100
Control solution/FCA	Intraperitoneal	100	100	80	80	80
Non-immunized infected	N/A[c]	100	100	100	100	80

[a] Freund's incomplete adjuvant
[b] Freund's complete adjuvant
[c] not applicable

Table 5-4. Prevalence of infection in fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi*.

		TIME (days postinfection)			
Treatment	Inoculum route	7	14	28	56
Whole dead	Intravenous	60	80	100	80
Control solution	Intravenous	100	100	100	67
Whole dead	Intramuscular	100	100	100	100
Control solution	Intramuscular	100	100	100	60
Whole dead	Intraperitoneal	100	100	100	100
Control solution	Intraperitoneal	75	100	100	67
Whole dead/FIA[a]	Intraperitoneal	100	100	100	75
Control solution/FIA[b]	Intraperitoneal	100	100	100	100
Whole dead/FCA	Intraperitoneal	80	100	100	60
Control solution/FCA	Intraperitoneal	100	100	100	75
Non-immunized infected	N/A[c]	100	100	100	75

[a] Freund's incomplete adjuvant

[b] Freund's complete adjuvant

[c] not applicable

Table 5-5. Abundance of parasites in fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi.*

Treatment	Inoculum route	TIME (days postinfection) 7	14	28	56
Whole dead	Intravenous	3.7 ± 1.57[a]	4.6 ± 1.27	4.7 ± 1.16	3.3 ± 0.89
Control solution	Intravenous	6.2 ± 0.82	7.1 ± 0.59	5.7 ± 0.98	3.2 ± 1.81
Whole dead	Intramuscular	7.2 ± 0.37	7.0 ± 0.16	6.6 ± 0.43	4.2 ± 0.97
Control solution	Intramuscular	6.5 ± 0.24	6.4 ± 0.89	6.3 ± 1.00	2.7 ± 1.42
Whole dead	Intraperitoneal	5.8 ± 0.18	5.6 ± 0.52	6.3 ± 0.29	3.9 ± 1.17
Control solution	Intraperitoneal	5.1 ± 1.82	3.4 ± 1.31	3.9 ± 0.76	1.8 ± 0.90
Whole dead/FIA[b]	Intraperitoneal	5.0 ± 0.94	6.6 ± 0.59	6.1 ± 0.38	4.3 ± 1.46
Control solution/FIA[c]	Intraperitoneal	7.5 ± 0.30	7.2 ± 0.18	7.1 ± 0.42	5.7 ± 0.14
Whole dead/FCA	Intraperitoneal	5.3 ± 1.35	6.0 ± 0.64	5.5 ± 0.96	3.6 ± 1.49
Control solution/FCA	Intraperitoneal	6.7 ± 0.45	6.7 ± 0.33	6.7 ± 0.54	4.1 ± 1.38
Non-immunized infected	N/A[d]	5.8 ± 0.96	7.0 ± 0.48	7.4 ± 0.41	4.4 ± 1.69

[a] mean number of parasites ml^{-1} blood ($\log_{10}$) ± SEM
[b] and [c], Freund's incomplete and complete adjuvant, respectively
[d] not applicable

Table 5-6. Correlation coefficient (r) between parasitemia and the antibody response of fish immunized with whole dead (sodium perchlorate-treated) *Trypanosoma danilewskyi.*

Treatment	Administration Route	r	*P*-value
Whole dead	Intravenous	0.01	N.S.[a]
Control solution	Intravenous	0.16	N.S.
Whole dead	Intramuscular	0.03	N.S.
Control solution	Intramuscular	0.26	N.S.
Whole dead	Intraperitoneal	0.41	N.S.
Control solution	Intraperitoneal	0.37	N.S.
Whole dead/FIA[b]	Intraperitoneal	0.83	$P < 0.0001$
Control solution/FIA[c]	Intraperitoneal	0.33	N.S.
Whole dead/FCA	Intraperitoneal	0.29	N.S.
Control solution/FCA	Intraperitoneal	0.70	$P < 0.006$
Non-immunized infected	N/A[d]	0.02	N.S.

[a] not significant
[b] Freund's incomplete adjuvant
[c] Freund's complete adjuvant
[d] not applicable

Table 5-7. Relative antibody response of fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi*.

Treatment	Inoculum route	TIME (days postinfection)				
		0	7	14	28	56
Whole dead	Intravenous	0.05 ± 0.022[a]	0.02 ± 0	0.07 ± 0.024	0.05 ± 0.021	0.05 ± 0.012
Control solution	Intravenous	0.02 ± 0.005	0.02 ± 0.013	0.03 ± 0.011	0.01 ± 0.004	0.01 ± 0.004
Whole dead	Intramuscular	0.02 ± 0.005	0.03 ± 0.011	0.05 ± 0.032	0.02 ± 0.018	0 ± 0.002
Control solution	Intramuscular	0.01 ± 0.004	0.02 ± 0.010	0.04 ± 0.006	0.01 ± 0.003	0.02 ± 0.008
Whole dead	Intraperitoneal	0.04 ± 0.013	0.04 ± 0.018	0.07 ± 0.015	0.04 ± 0.008	0.04 ± 0.004
Control solution	Intraperitoneal	0.01 ± 0.002	0 ± 0	0.03 ± 0.007	0.01 ± 0.002	0.02 ± 0.008
Whole dead/FIA[b]	Intraperitoneal	0.06 ± 0.009	0.05 ± 0.014	0.08 ± 0.031	0.04 ± 0.015	0.03 ± 0.017
Control solution/FIA[c]	Intraperitoneal	0.02 ± 0.008	0.02 ± 0.007	0.05 ± 0.015	0.05 ± 0.006	0.03 ± 0.006
Whole dead/FCA	Intraperitoneal	0.04 ± 0.017	0.02 ± 0.011	0.09 ± 0.022	0.05 ± 0.024	0.06 ± 0.016
Control solution/FCA	Intraperitoneal	0.03 ± 0.014	0.01 ± 0.010	0.03 ± 0.018	0.02 ± 0.010	0.03 ± 0.012
Non-immunized:infected	N/A[d]	0.04 ± 0.011	0.03 ± 0.009	0.05 ± 0.010	0.03 ± 0.013	0.02 ± 0.006

[a] mean ELISA value ($\log_{10}$) ± SEM
[b] Freund's incomplete adjuvant
[c] Freund's complete adjuvant
[d] not applicable

Table 5-8. Percent of control value[a] of the antibody response of fish immunized with whole dead (sodium perchlorate-treated) *Trypanosoma danilewskyi*.

	TIME (days postinfection)			
Administration route	7	14	28	56
Intravenous	N.D.[b]	87	234	113
Intramuscular	27	46	154	4
Intraperitoneal	N.D.	90	118	71
Intraperitoneal/FIA[c]	133	4	1	1
Intraperitoneal/FCA[d]	46	101	65	83

[a] ELISA values transformed into the percent increase or decrease of that observed 0 dpi, which equals 100%

[b] not done

[c] Freund's incomplete adjuvant

[d] Freund's complete adjuvant

Table 5-9. Red cell volume (%) in blood of fish given sodium-perchlorate-treated trypanosomes or control solution prior to challenge with *Trypanosoma danilewskyi.*

		TIME (days postinfection)				
Treatment	Inoculum route	0	7	14	28	56
Whole dead	Intravenous	40.2 ± 1.77[a]	30.0 ± 0	37.2 ± 2.06	38.8 ± 0.74	40.0 ± 1.10
Control solution	Intravenous	44.8 ± 1.88	35.7 ± 2.33	35.2 ± 1.20	35.7 ± 1.86	39.3 ± 1.33
Whole dead	Intramuscular	37.8 ± 0.58	34.7 ± 0.33	36.0 ± 1.73	35.7 ± 2.40	38.3 ± 1.76
Control solution	Intramuscular	40.6 ± 2.50	39.7 ± 4.26	36.6 ± 2.25	33.4 ± 2.38	37.8 ± 2.06
Whole dead	Intraperitoneal	40.2 ± 2.31	37.3 ± 4.68	37.0 ± 1.82	36.2 ± 3.85	32.0 ± 4.95
Control solution	Intraperitoneal	35.5 ± 1.85	35.0 ± 4.00	30.7 ± 1.45	34.7 ± 0.33	34.0 ± 0.58
Whole dead/FIA[b]	Intraperitoneal	35.4 ± 1.63	38.0 ± 2.65	31.4 ± 4.67	36.0 ± 1.41	36.5 ± 2.26
Control solution/FIA[c]	Intraperitoneal	37.4 ± 2.23	31.3 ± 1.67	32.3 ± 2.40	23.0 ± 4.93	32.7 ± 5.84
Whole dead/FCA	Intraperitoneal	40.2 ± 1.77	37.7 ± 1.86	37.2 ± 1.36	37.6 ± 2.04	36.4 ± 2.71
Control solution/FCA	Intraperitoneal	40.0 ± 2.78	39.5 ± 1.50	33.0 ± 2.27	28.8 ± 3.95	29.8 ± 2.69
Non-immunized infected	N/A[d]	39.5 ± 2.63	39.0 ± 2.42	34.4 ± 3.31	27.2 ± 4.59	35.7 ± 1.67

[a] mean red cell volume ± SEM
[b] Freund's incomplete adjuvant
[c] Freund's complete adjuvant
[d] not applicable

Table 5-10. Summary of results (parasitemia) obtained from experiments that evaluated the efficacy of parasite extracts and inoculum route on the susceptibility of goldfish to *Trypanosoma danilewskyi*.

Treatment	Inoculum route	0 to 7 dpi[a]	14 to 56 dpi
Whole dead	Intravenous	N.S.[b]	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S	**$P < 0.03$**[e]
	with FIA[c]	$P < 0.04$	N.S.
	with FCA[d]	N.S.	N.S.
Trypanosome lysate	Intravenous	N.S.	**$P < 0.04$**
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	N.S.	N.S.
	with FCA	N.S.	N.S.
Water-soluble extract	Intravenous	N.S.	N.S.
	Intraperitoneal	N.S.	**$P < 0.04$**
	with FIA	N.S.	N.S.
	with FCA	N.S.	N.S.
Detergent-soluble extract	Intravenous	N.S.	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	**$P < 0.02$**	N.S.
	with FCA	N.S.	N.S.
ES product[f]	Intravenous	N.S.	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	N.S.	N.S.
	with FCA	$P < 0.008$	N.S.

[a] days postinfection
[b] not significant
[c] Freund's incomplete adjuvant
[d] Freund's complete adjuvant
[e] boldface type indicates that parasitemia was enhanced by immunization
[f] excretory-secretory product

Table 5-11. Summary of results (parasitemia) comparing the susceptibility of goldfish, administered control solution prior to *Trypanosoma danilewskyi* infection, with that of non-immunized infected goldfish.

Control solution of antigen	Inoculum route	0 to 7 dpi[a]	14 to 56 dpi
Whole dead	Intravenous	N.S.[b]	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	$P < 0.003$
	with FIA[c]	N.S.	N.S.
	with FCA[d]	N.S.	N.S.
Trypanosome lysate	Intravenous	N.S.	$P < 0.0001$
	Intramuscular	N.S.	$P < 0.0006$
	Intraperitoneal	N.S.	$P < 0.03$
	with FIA	N.S.	$P < 0.003$
	with FCA	N.S.	$P < 0.02$
Water-soluble extract	Intravenous	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	N.S.	N.S.
	with FCA	N.S.	N.S.
Detergent-soluble extract	Intravenous	N.S.	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	$P < 0.04$	$P < 0.008$
	with FCA	$P < 0.02$	N.S.
ES product[e]	Intravenous	N.S.	$P < 0.0002$
	Intramuscular	N.S.	$P < 0.02$
	Intraperitoneal	N.S.	$P < 0.05$
	with FIA	N.S.	$P < 0.006$
	with FCA	N.S.	$P < 0.01$

[a] days postinfection
[b] not significant
[c] Freund's incomplete adjuvant
[d] Freund's complete adjuvant
[e] excretory-secretory product

Table 5-12. Summary of results (ELISA values) obtained from experiments that evaluated the efficacy of immunization on the humoral immune response of goldfish, prior to infection with *Trypanosoma danilewskyi*.

Treatment	Inoculum route	0 dpi[a]
Whole dead	Intravenous	N.S.[b]
	Intramuscular	N.S.
	Intraperitoneal	**$P < 0.05$**
	with FIA[c]	**$P < 0.025$**
	with FCA[d]	N.S.
Trypanosome lysate	Intravenous	N.S.
	Intramuscular	N.S.
	Intraperitoneal	N.S.
	with FIA	N.S.
	with FCA	N.S.
Water-soluble extract	Intravenous	$P < 0.05$
	Intraperitoneal	**$P < 0.05$**
	with FIA	N.S.
	with FCA	N.S.
Detergent-soluble extract	Intravenous	N.S.
	Intramuscular	N.S.
	Intraperitoneal	N.S.
	with FIA	N.S.
	with FCA	N.S.
ES product[f]	Intravenous	N.S.
	Intramuscular	N.S.
	Intraperitoneal	N.S.
	with FIA	N.S.
	with FCA	N.S.

[a] days postinfection
[b] not significant
[c] Freund's incomplete adjuvant
[d] Freund's complete adjuvant
[e] boldface type indicates that ELISA value was enhanced by immunization
[f] excretory-secretory product

Table 5-13. Summary of results (ELISA values) obtained from experiments that evaluated the efficacy of parasite extracts and inoculum route on the susceptibility of goldfish to *Trypanosoma danilewskyi.*

		TIME (days postinfection)				
Treatment	Inoculum route	4	7	14	28	56
Whole dead	Intravenous	N.D.[a]	N.D.	N.S.[b]	N.S.	N.S.
	Intramuscular	N.D.	N.S.	N.S.	N.S.	N.S.
	Intraperitoneal	N.D.	N.D.	N.S.	N.S.	N.S.
	with FIA[c]	N.D.	N.S.	N.S.	N.S.	N.S.
	with FCA[d]	N.D.	N.S.	N.S.	N.S.	N.S.
Trypanosome lysate	Intravenous	N.D.	**$P<0.05$**[e]	N.S.	N.S.	N.S.
	Intramuscular	N.D.	N.S.	N.S.	N.S.	N.S.
	Intraperitoneal	N.D.	N.S.	N.S.	N.S.	N.S.
	with FIA	N.D.	N.S.	N.S.	N.S.	N.S.
	with FCA	N.D.	N.S.	N.S.	N.S.	N.S.
Water-soluble extract	Intravenous	N.S.	N.S.	N.S.	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.	N.S.	N.S.	N.S.
	with FIA	N.S.	N.S.	N.S.	N.S.	N.S.
	with FCA	N.S.	N.S.	N.S.	N.S.	N.S.
ES product[f]	Intravenous	N.D.	N.S.	N.S.	N.S.	N.S.
	Intramuscular	N.D.	N.S.	N.S.	$P<0.03$	$P<0.05$
	Intraperitoneal	N.D.	N.S.	N.S.	N.S.	N.S.
	with FIA	N.D.	N.S.	N.S.	N.S.	N.S.
	with FCA	N.D.	N.S.	N.S.	N.S.	N.S.

[a] not done
[b] not significant
[c] Freund's incomplete adjuvant
[d] Freund's complete adjuvant
[e] boldface type indicates that ELISA value was enhanced by immunization
[f] excretory-secretory product

Table 5-14. Summary of results (red cell volume) obtained from experiments that evaluated the efficacy of parasite extracts and inoculum route on the susceptibility of goldfish to *Trypanosoma danilewskyi*.

Treatment	Inoculum route	0 to 7 dpi[a]	14 to 56 dpi
Whole dead	Intravenous	N.S.[b]	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA[c]	N.S.	N.S.
	with FCA[d]	N.S.	**$P < 0.03$**
Trypanosome lysate	Intravenous	N.S.	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	N.S.	N.S.
	with FCA	N.S.	N.S.
Water-soluble extract	Intravenous	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	N.S.	N.S.
	with FCA	N.S.	N.S.
Detergent-soluble extract	Intravenous	N.S.	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	**$P < 0.02$**
	with FIA	**$P < 0.03$**	**$P < 0.008$**
	with FCA	N.S.	N.S.
ES product[f]	Intravenous	N.S.	N.S.
	Intramuscular	N.S.	N.S.
	Intraperitoneal	N.S.	N.S.
	with FIA	N.S.	N.S.
	with FCA	N.S.	$P < 0.02$

[a] days postinfection
[b] not significant
[c] Freund's incomplete adjuvant
[d] Freund's complete adjuvant
[e] boldface type indicates that red cell volume of immunized group was greater
[f] excretory-secretory product

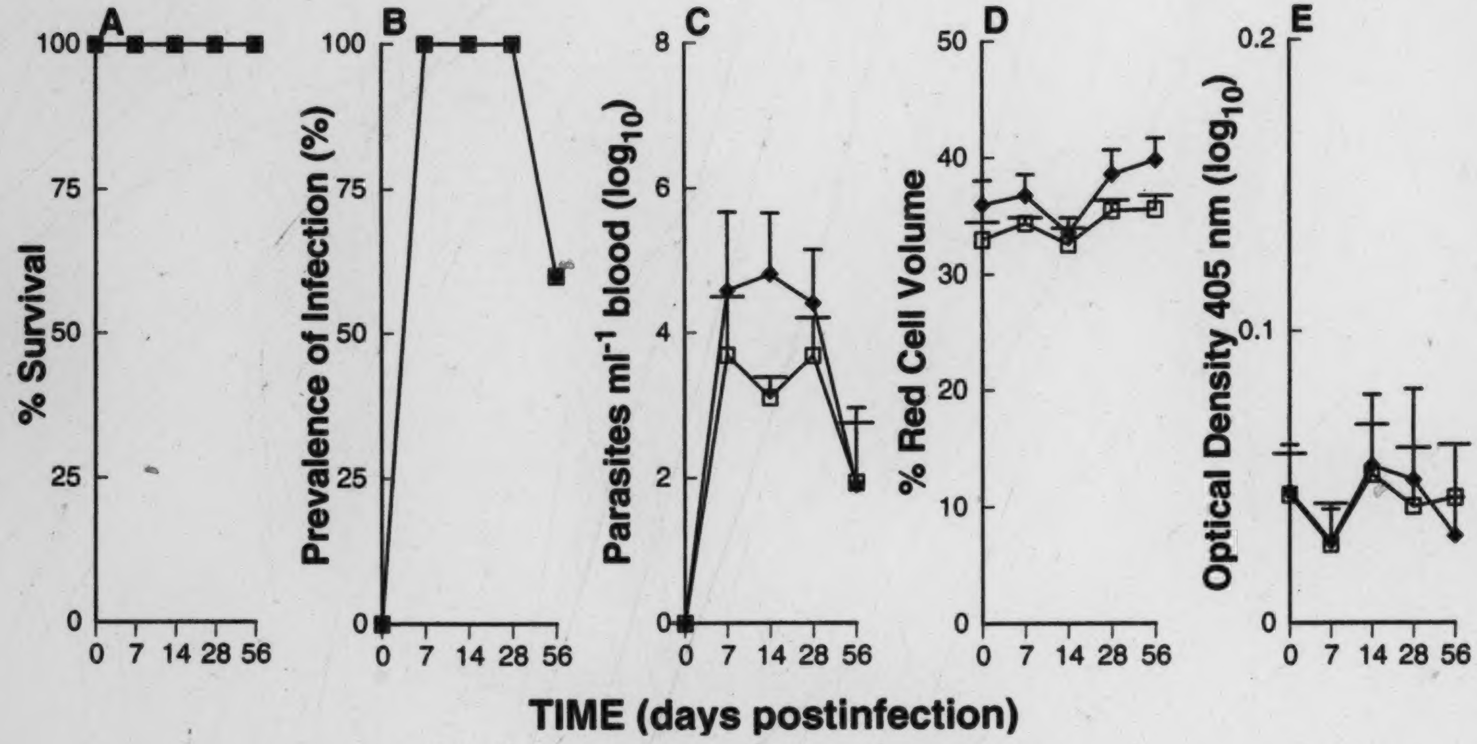

Fig. 5-1. Effect of intravenous administration of ◆ trypanosome excretory-secretory products or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

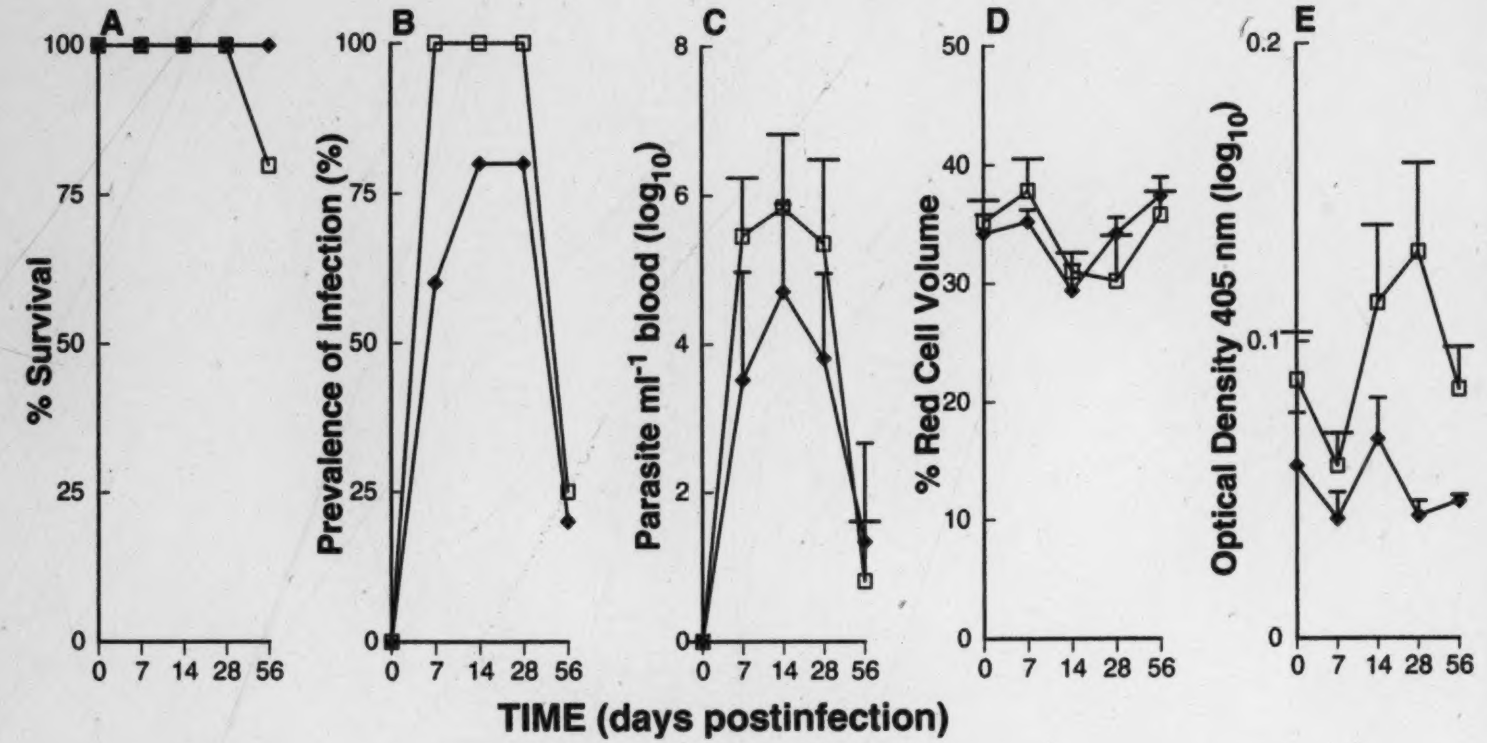

Fig. 5-2. Effect of intramuscular administration of ◆ trypanosome excretory-secretory products or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

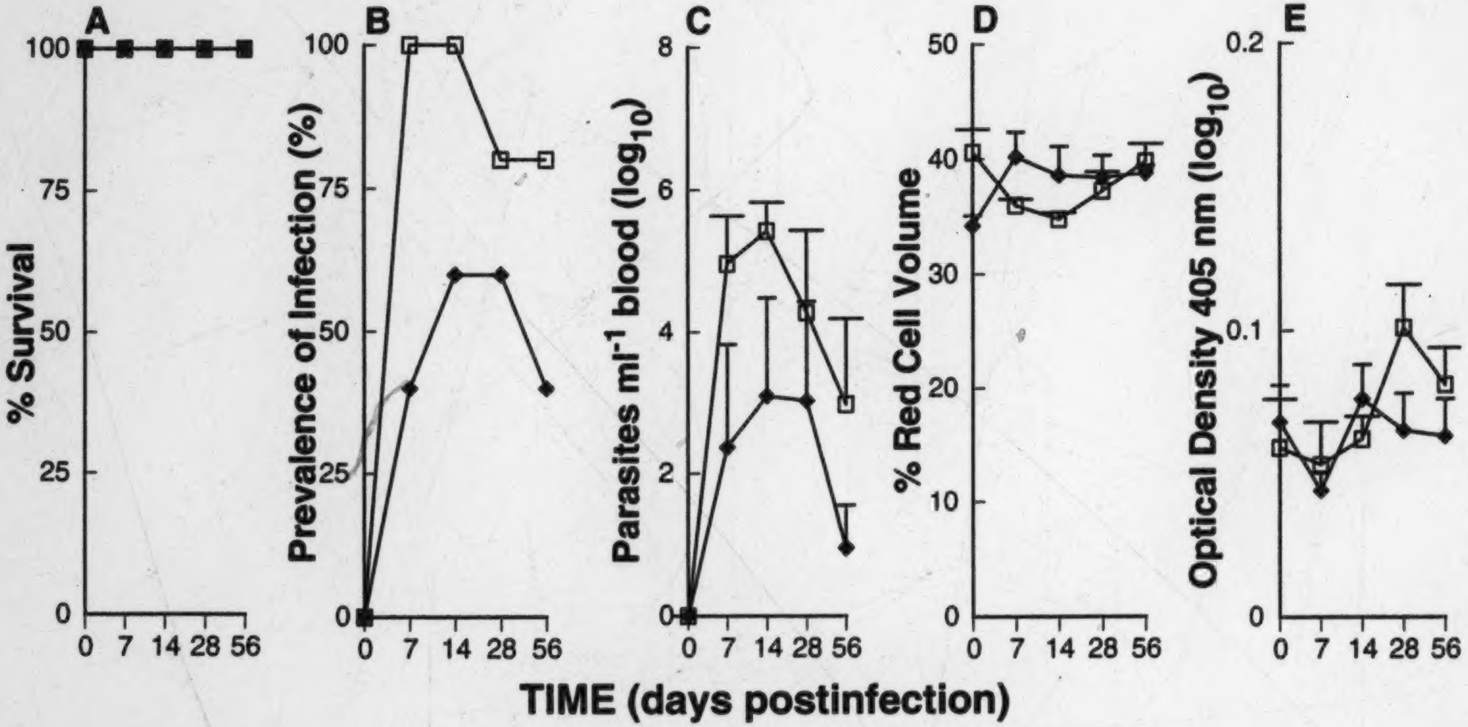

Fig. 5-3. Effect of intraperitoneal administration of ◆ trypanosome excretory-secretory products or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

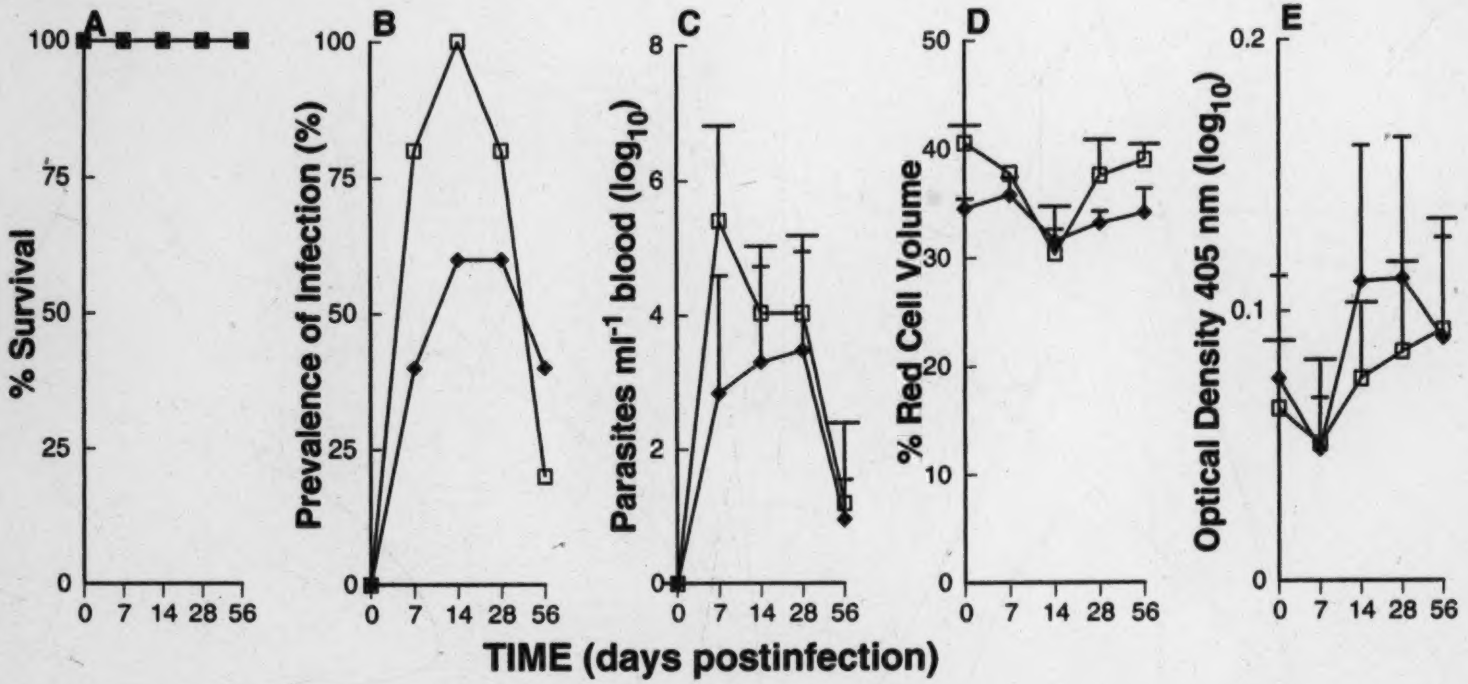

Fig. 5-4. Effect of intraperitoneal administration of ◆ trypanosome excretory-secretory products/Freund's incomplete adjuvant or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

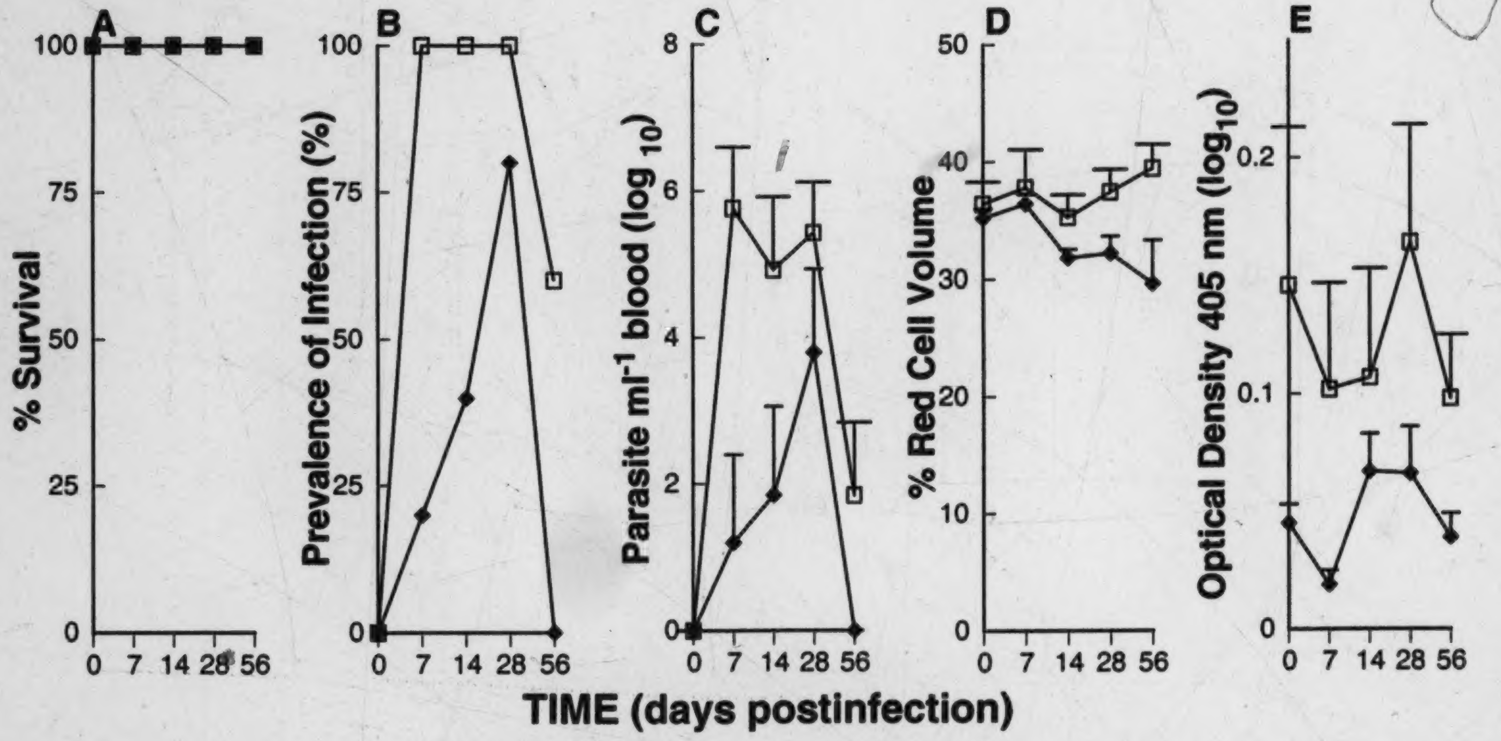

Fig. 5-5. Effect of intraperitoneal administration of ◆ trypanosome excretory-secretory products/Freund's complete adjuvant or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

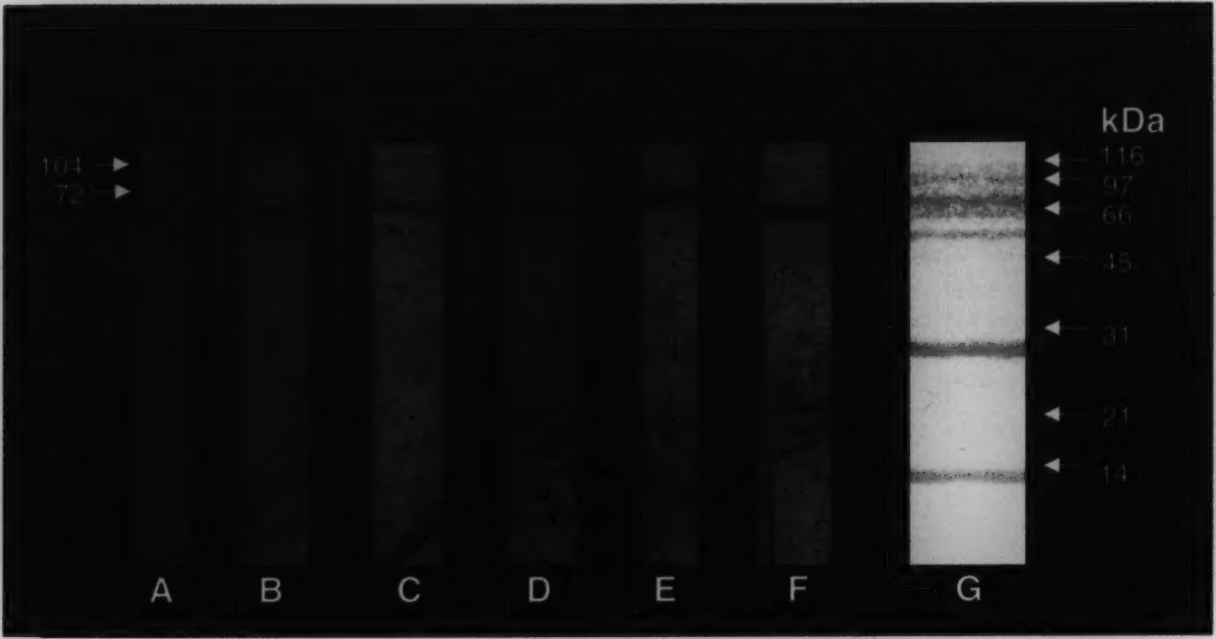

Fig. 5-7. Immunoblot of *Trypanosoma danilewskyi* excretory-secretory products probed with serum from immune (lanes a to c) and normal (lanes d to f) fish. Sera were diluted 1:10 (lane a and d), 1:25 (lane b and e), and 1:50 (lane c and f). Coomassie stain of excretory-secretory products (lane g). Each lane contained products from 1.5 X 10^7 trypanosomes.

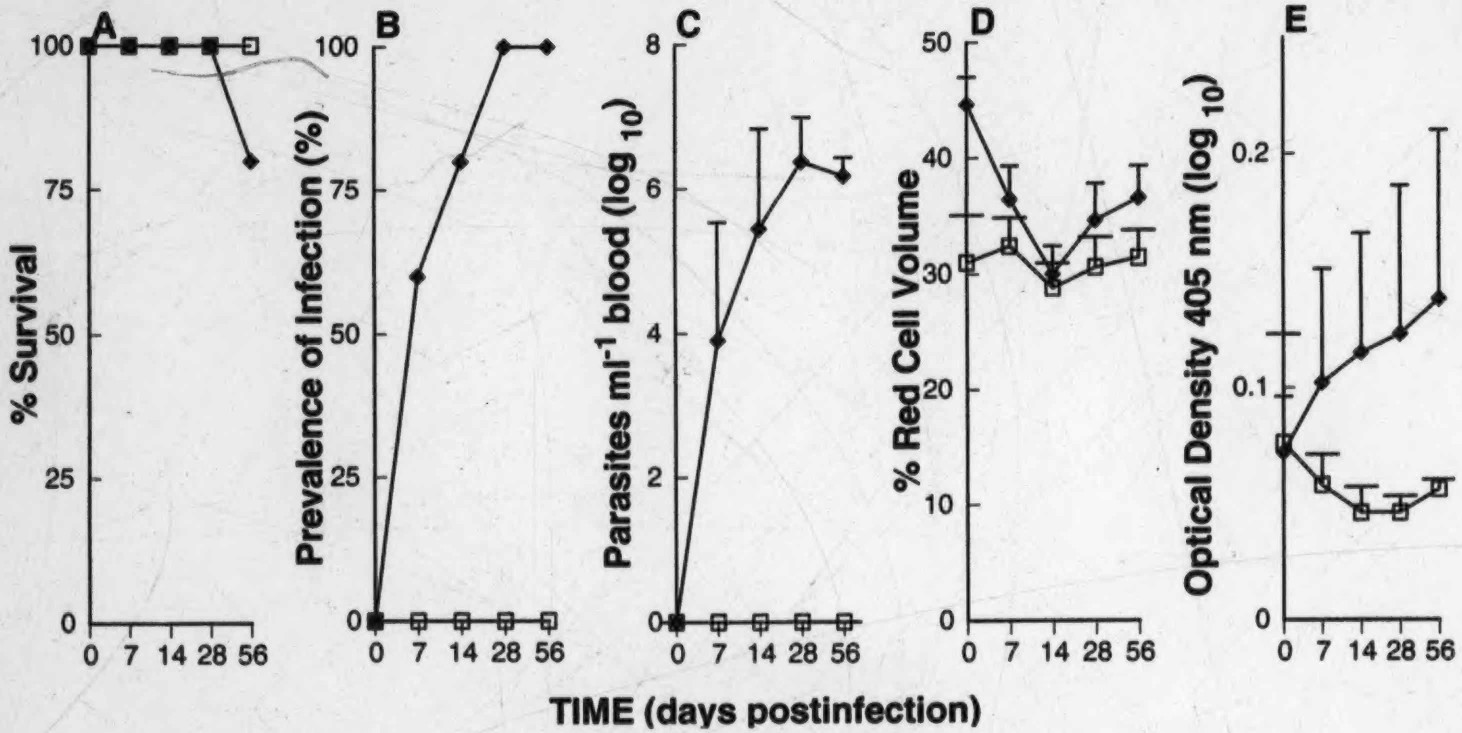

Fig. 5-7. Infection dynamics of non-immunized goldfish (◆ inoculated with *Trypanosoma danilewskyi* or ☐ uninfected) included in the trypanosome excretory-secretory product immunization experiment. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

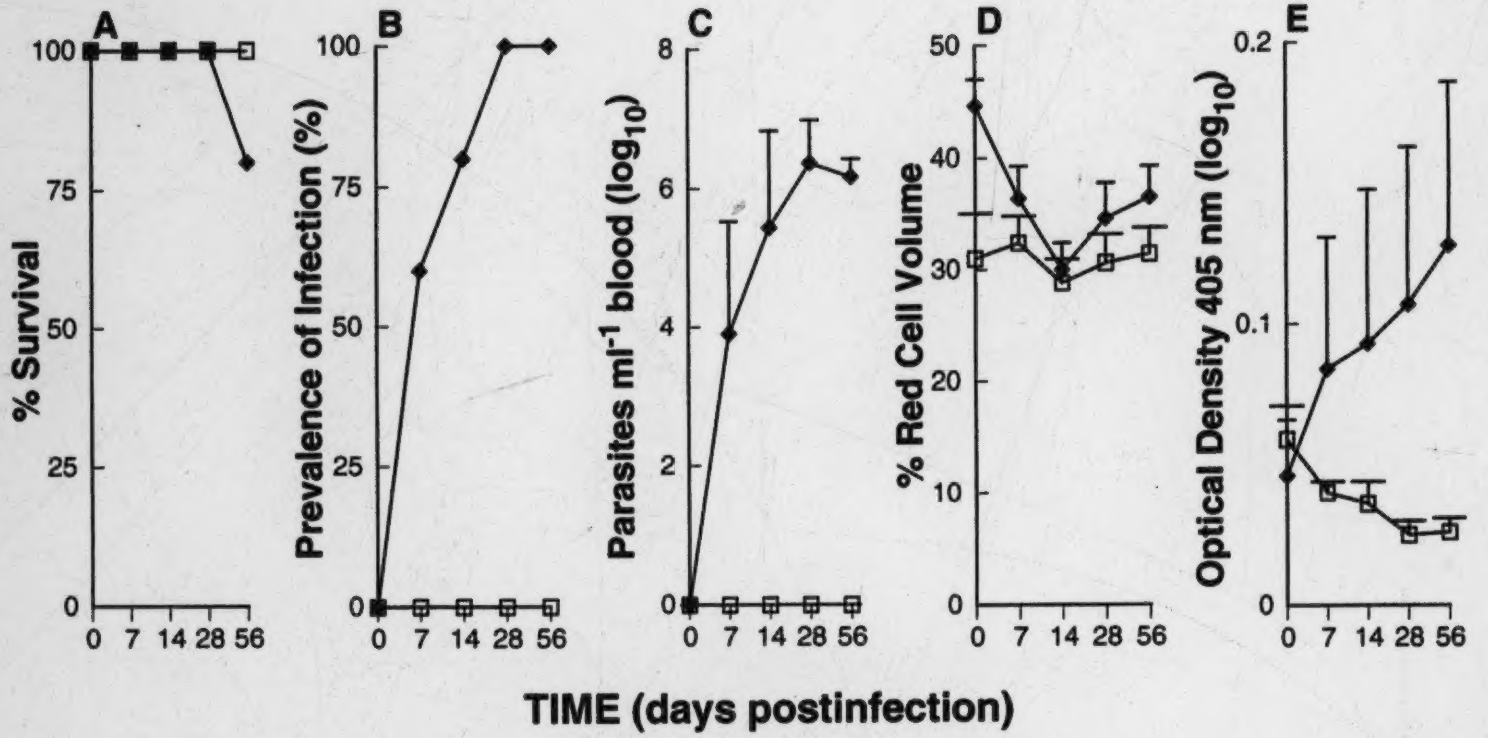

Fig. 5-8. Infection dynamics of non-immunized goldfish (◆ inoculated with *Trypanosoma danilewskyi* or ☐ uninfected) included in the trypanosome lysate immunization experiment. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

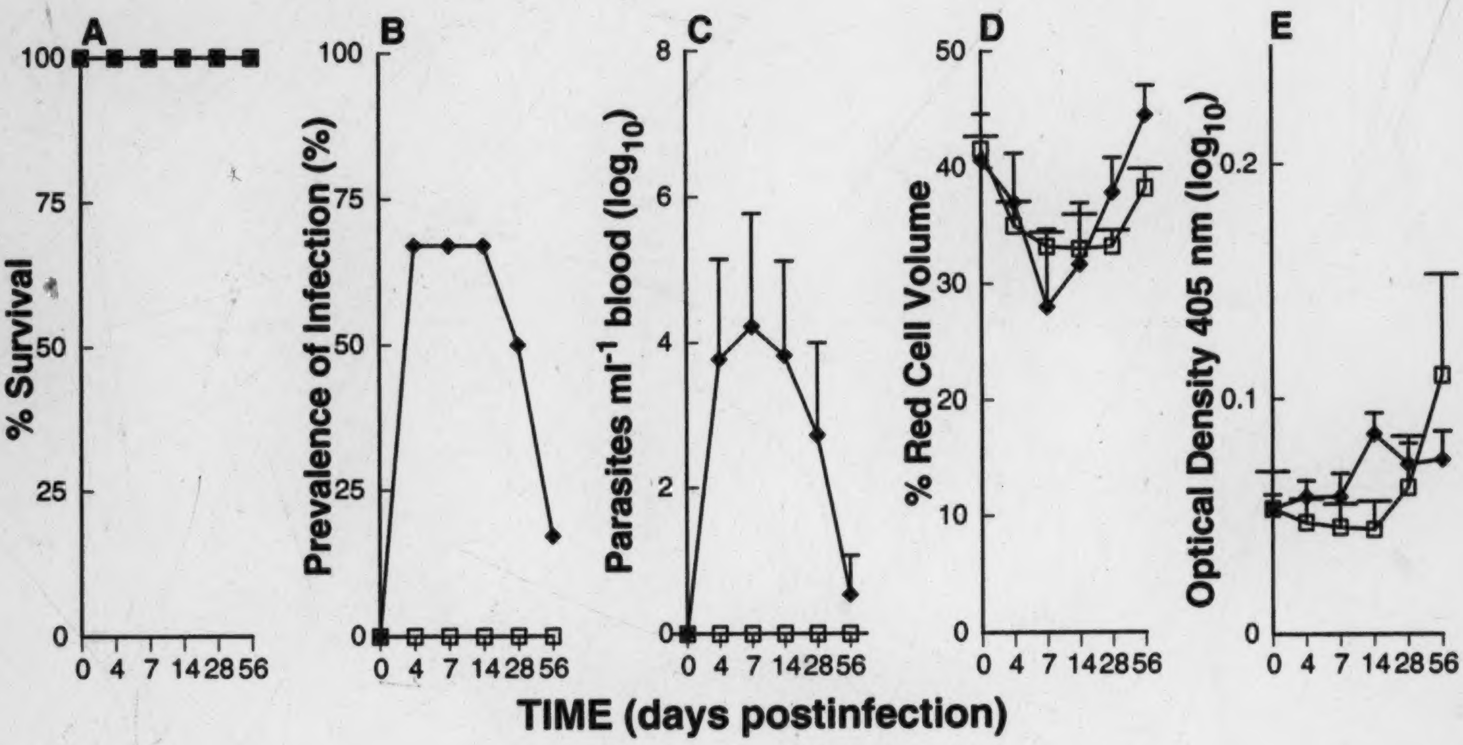

Fig. 5-9. Infection dynamics of non-immunized goldfish (◆ inoculated with *Trypanosoma danilewskyi* or □ uninfected) included in the water-soluble molecule immunization experiment. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

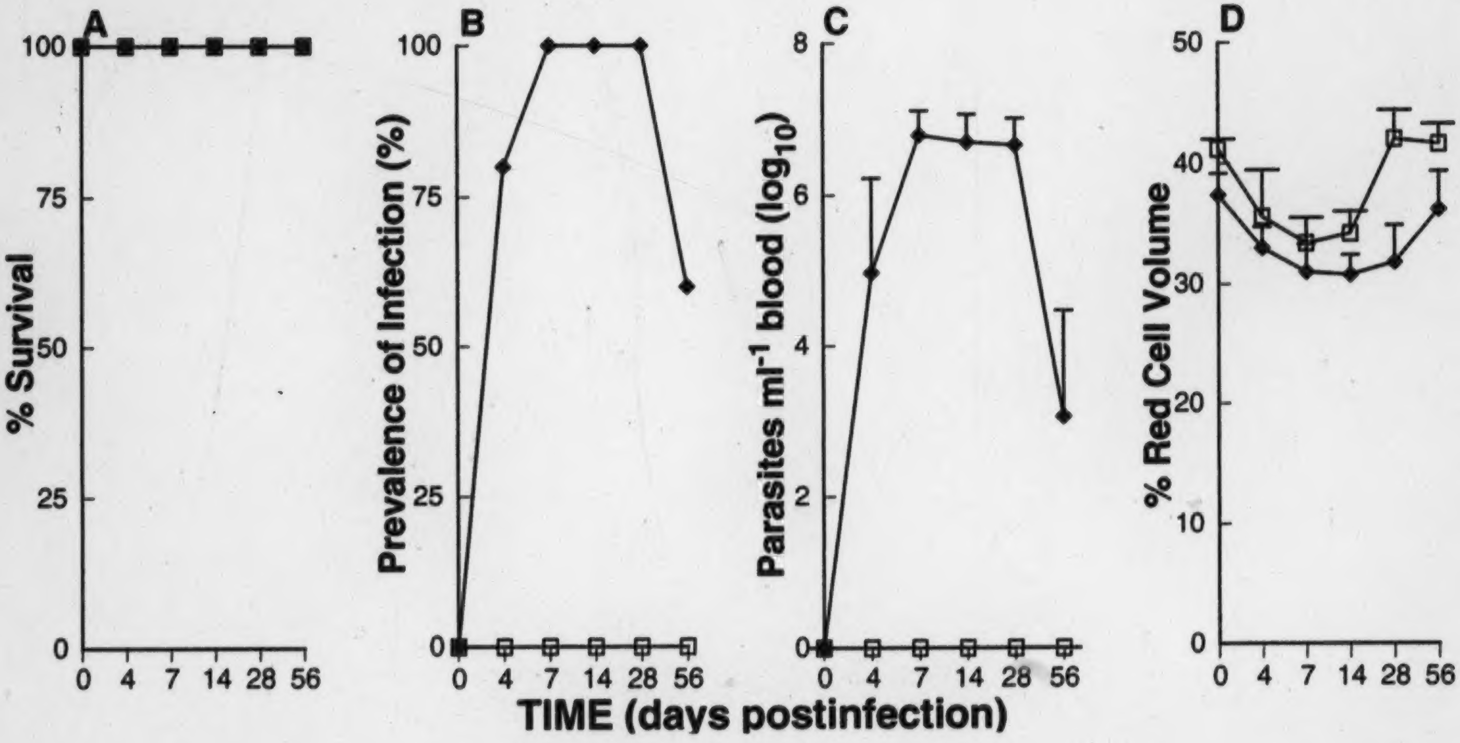

Fig. 5-10. Infection dynamics of non-immunized goldfish (◆ inoculated with *Trypanosoma danilewskyi* or ☐ uninfected) included in the detergent-soluble molecule immunization experiment. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells. In figures C and D, data points represent the mean ± SEM.

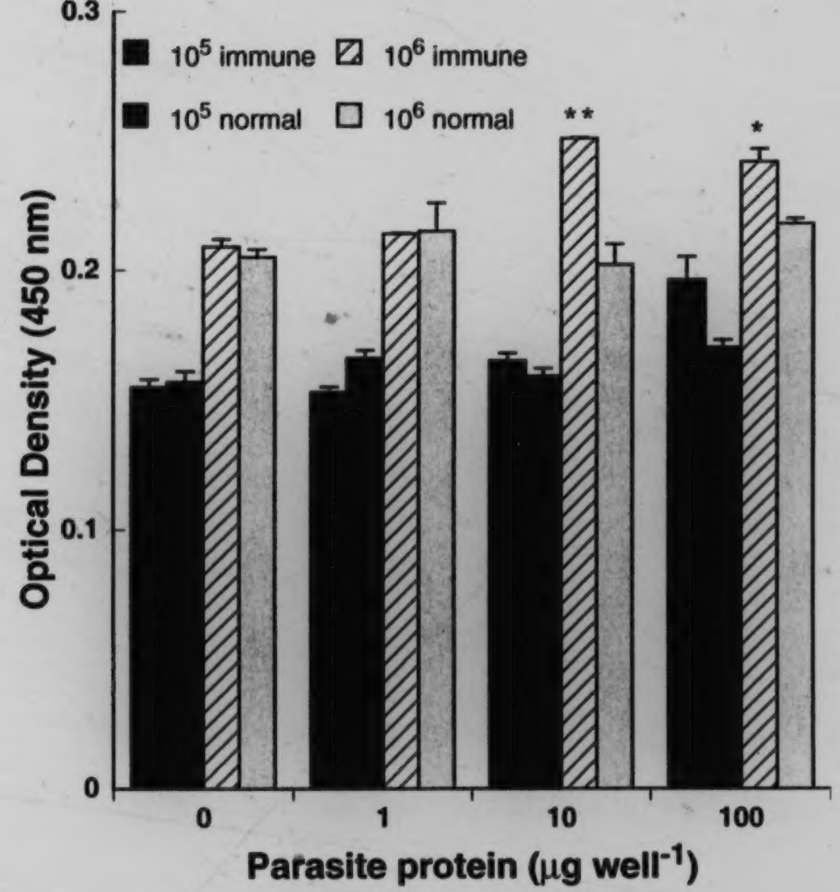

Fig. 5-11. Antigen-induced responsiveness of peripheral blood lymphocytes, isolated from immune (10^5, or 10^6 cells well^{-1}) or naïve (10^5, or 10^6 cells well^{-1}) fish, that were incubated with trypanosome lysate. Data points represent the mean optical density ± SEM (n = 3). $P < 0.002$ and $P < 0.0001$ (* and **, respectively) when compared to nonimmune cells (10^6 cells well^{-1}) stimulated with 10 or 100 µg ml^{-1} of parasite protein.

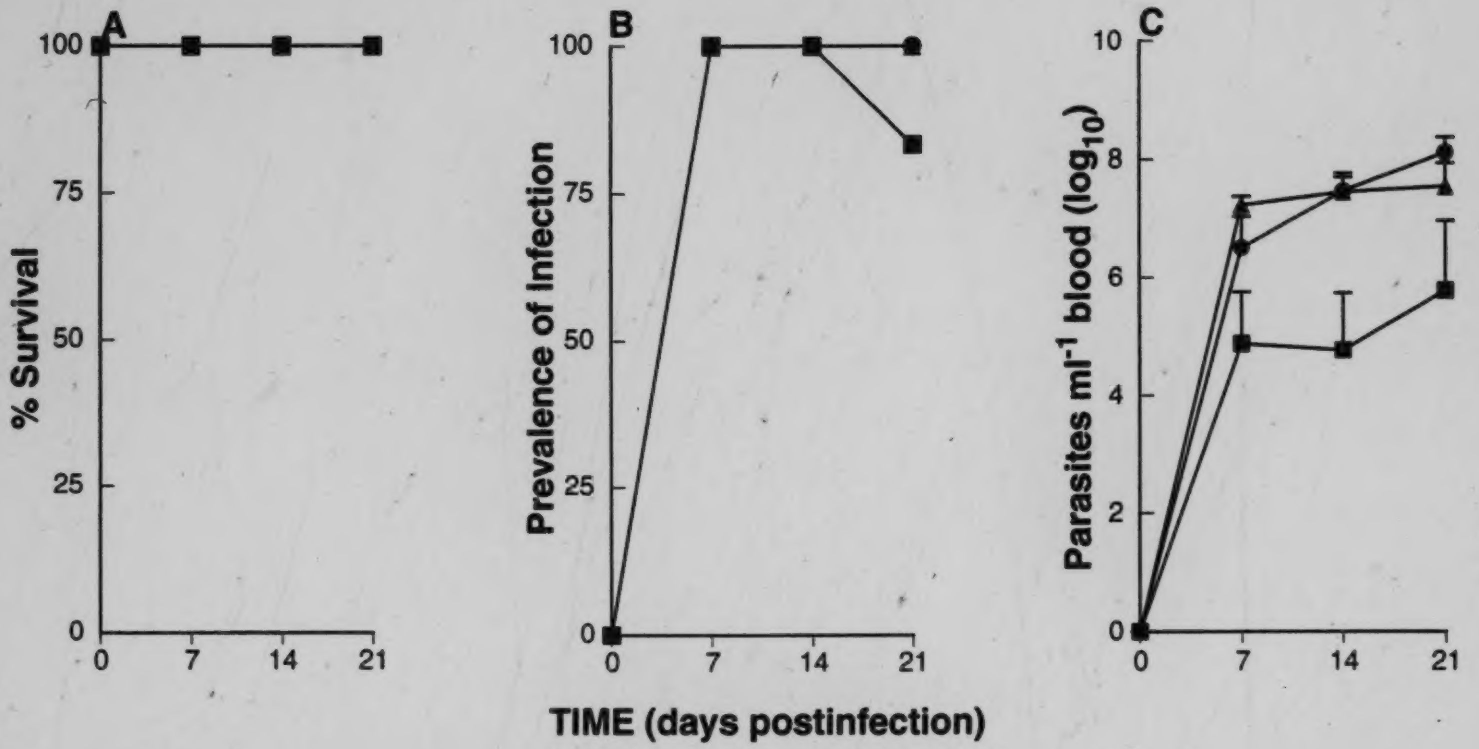

Fig. 5-12. Mortality (A), prevalence of infection (B), and course of infection (C) in naïve goldfish (n=6) that were immunized with ■ immune or ● normal serum. Goldfish not immunized (▲) prior to inoculation with *Trypanosoma danilewskyi*. Data points in 5-30C represent the mean number of parasites ml^{-1} blood ± SEM.

CHAPTER VI

GENERAL DISCUSSION

This study was undertaken to augment our knowledge of the biological and immunological relationship between *T. danilewskyi* and its vertebrate hosts. One objective included establishing conditions that are necessary for *in vitro* cultivation. The following conclusions may be drawn from the *in vitro* cultivation experiments: 1) serum-related factors within fish blood are requisite for long-term cultivation of *T. danilewskyi*; 2) the generation time of trypanosomes can be manipulated by the amount of fish serum and FBS present in the medium; 3) trypanosomes cultured in 10% GFS could be used as a source of antigen in immunoassays and for establishing a primary infection, as > 80% of the cultures consisted of bloodstream forms and metacyclic trypomastigotes. Further, *in vitro* growth of *T. danilewskyi* is facilitated by the presence of living cells or cell-conditioned medium. Growth enhancing activity of cell-conditioned medium is not specific to the species (mouse, human, or goldfish), immune status of the fish (naïve, infected, or recovered), or cell-type (macrophage, monocyte, or fibroblast). Cell products, used for the enhancement of growth, appear to be required in small quantities, as a negative correlation between total protein content and parasite number was consistently drawn. Additionally, dilution (50% v/v) of cell-conditioned medium did not adversely affect growth-enhancing activity.

For immunological and biochemical studies on *T. danilewskyi*, it would be advantageous to have a defined medium with components that are readily available. For unknown reasons, serum-free medium described by Wang and Belosevic [284] was only able to sustain the parasite cultures for a few days [284]. My work and a subsequent study demonstrated that *T. danilewskyi* required fish serum for long-term *in vitro* cultivation [35, 216]. Unfortunately, maintenance of a fish colony as serum donors is a costly and laborious undertaking. Notwithstanding, supplementing fish serum with commercially available bovine serum can minimize the requirement of fish serum. Also, it was fortunate that serum from other cyprinids, which grow faster and larger than goldfish, supported *T. danilewskyi in vitro*. At present, it is unknown which serum component(s) were required for long-term cultivation. Future studies, which test fractionated serum (i.e., size-exclusion, or salting-out), may result in a more simplified medium.

Another important aspect of cultivation was to determine if cultured parasites were morphologically and antigenically similar to the stages present in the bloodstream of fish. Results from my morphometric study indicated that the predominant life-cycle stage in the cultures was the bloodstream-form. Further, other workers have illustrated that trypanosomes cultivated under similar conditions were antigenically similar.

Although data collected from the experiments, using cell-conditioned medium, could be adapted to maintain *T. danilewskyi* in the absence of fish serum, my intention was to further elucidate the biological relationship that has been established between cyprinids and blood-borne diseases. Particularly, these experiments were designed to determine: 1) whether trypanosomes regulated the production of growth-enhancing factors by host cells; 2) if supportive activity was conserved between species; and 3) the role of cells, which would be closely associated with the inflammation and tissue repair.

Supernatants, collected from macrophage or fibroblast cultures, enhanced the *in vitro* growth of *T. danilewskyi*. Although not confirmed by *in vivo* studies, it seems unlikely that this phenomena was limited to culturing conditions, as a histological study demonstrated that a massive number of trypanosomes aggregated in the haematopoietic organs [81]. Additionally, results from my experiments and others [215] suggested that trypanosomes were present in the kidney long after the parasites were cleared from the peripheral blood. Using this information, it seems reasonable to propose the following relationship between goldfish and *T. danilewskyi*. Early in the infection, *T. danilewskyi* infiltrates the haematopoietic organs, causing severe histopathological changes. As a result, the host is stressed, the protective responses are undermined, and an inflammatory response is evoked at the site of the tissue damage. The neutrophils and macrophages, which are involved in the inflammatory response, attempt to control the infection (discussed later), while simultaneously producing soluble products that serve as growth-enhancing factors for *T. danilewskyi*. Conceivably, as the infection is reduced, the inflammatory response is limited and there is an induction of tissue repair and regeneration. During the healing process, capillaries grow, fibroblasts accumulated, scar tissue is formed, and the trypanosomes derive nutrition from the associated connective tissue cells. After recovery from the acute infection, small numbers of trypanosomes reside in the haematopoietic organs, deriving nutrition from serum components and resident macrophages. In accordance with this proposed relationship, it would be interesting to determine the effect of other mediators of inflammation and tissue repair (i.e., neutrophils, eosinophils, basophils, parenchymal cells, fibrin, etc.) on the growth of *T. danilewskyi*.

The role of phagocytes in *T. danilewskyi* infection appears paradoxical. Of all the killing mechanisms, only anecdotal evidence of phagocytosis has been published [81]. A preliminary *in vitro* experiment, conducted with fish or mammalian macrophages, suggested that phagocytosis does not play a crucial role in recovering from *T. danilewskyi* infection (Appendix C). The effects of reactive oxygen or nitrogen intermediates have not been determined in this host-parasite system. Nevertheless, it seems reasonable to question the biological relevance of these mechanisms, inasmuch as *T. danilewskyi* persists in the kidney. (Interestingly, the phagocytes in the kidney of a cyprinid comprises ~13% of the total leukocyte population [244].) Clearly, future work is required to elucidate the role of non-specific defense mechanisms and accessory functions of phagocytes. With the little bit of information that is presently available, it may be of particular interest to study the regulation of macrophage defense mechanisms by live parasites and their products.

Growth-enhancing activity of fish macrophages was independent of the course of infection, as parasite growth in medium collected from cultures derived from infected fish did not differ markedly from those acquired from normal fish. Evidently, *T. danilewskyi* does not stimulate host cell secretion of growth-enhancing factors. If it were otherwise, low numbers of trypanosomes would not be as efficient in establishing an infection, as it would be likely that activation of host cells would be dependent on the concentration of the signal produced by the parasite.

As mentioned in Chapter I, *T. brucei* was believed to constitute the earliest divergent branch of *T. danilewskyi* [192]. This implied that the evolution of trypanosomes of terrestrial animals was accompanied by a secondary acquisition of aquatic hosts and habitats. This is supported by evidence obtained from cultivating *T. danilewskyi* in medium conditioned by mammalian cells. My studies indicated that species specificity of growth-enhancing activity was lacking, as *in vitro* growth of *T. danilewskyi* could be augmented by medium conditioned by mammalian cells. Although these experiments did not indicate that enhanced growth was caused by the selfsame molecule(s), they do suggest that trypanosomes use molecules that have been conserved over time. Another interesting aspect of this proposed phylogeny, was the survival of trypanosomes in the presence of host defense mechanisms. Briefly, *T. danilewskyi* evolved from trypanosomatids that survived in terrestrial vertebrate hosts, which presumably had a more sophisticated immune system than that present in primitive aquatic vertebrates. Under these circumstances, it would be understandable that a long-term infection establishes in fish.

As with medium supplemented with serum, it would be academically fascinating to identify the host molecule(s) that facilitate growth of *T. danilewskyi*. Nevertheless, the application of this knowledge for the development of drug targeting is unlikely, unless the relevant steps in the metabolic pathway are either absent from the host or differ from analogous steps in the host [267]. An additional concern is that the putative molecule must be involved in a single rate-limiting step. That is to say, if one metabolic pathway was blocked, the parasite has no other compensatory mechanisms. An excellent example of the difficulty of meeting the second criterion was illustrated in the purine metabolism of mammalian trypanosomes. Trypanosomes were not able to use precursors (i.e., glycine or serine) for the synthesis of nucleic acid purine bases [32, 57, 100]. Thereby suggesting that these protozoa were incapable of *de novo* synthesis of purines and rely exclusively on the salvage of preformed bases [32, 57, 207]. Nucleotides can be formed from purines and purine nucleosides. Nucleotides can also be formed from the conversion of purines into nucleotides and these to nucleotides. Nucleotides can be formed from nucleosides through the addition of a phosphate to the ribose moiety by purine kinases (reviewed by [122, 124, 191]). Further, the presence of enzymes of purine salvage in trypanosomes suggested that there were a plethora of salvage pathways. This situation becomes more complex in that pathways may differ between species. For example, *T. cruzi* and *T. congolense* can produce adenosine monophosphate (AMP) via adenosine phosphorylase or APRT (adenosine phosphoribosyltransferase). Of these enzymes, only APRT has been reported in *T. vivax*. However, adenine deaminase activity has been detected in the latter species, suggesting that converting adenine to hypoxanthine can indirectly produce AMP. Moreover, the presence of purine salvage enzymes appears to differ among trypanosome strains. Adenosine deaminase activity, for the interconversion of nucleosides, was present in the Sonya strain of *T. cruzi* [68, 122] but not in the Peru strain [32]. Likewise, *T. brucei* trypomastigotes (LSH14.2.1.64) possess adenosine phosphorylase, adenosine deaminase, and adenosine kinase activity [68], while the ILRAD 1203 line does not [209].

Included as part of the main objective, this study identified components of the host-parasite relationship that influenced the susceptibility of goldfish to *T. danilewskyi*. The following conclusions were drawn from these investigations.

Goldfish acquire resistance to *T. danilewskyi* after initial exposure and alleviation of the acute primary infection. Resistance to reinfection may be associated with non-sterile immunity, as very low numbers of parasites were detected in the blood and other tissues of recovered fish. Further, resistance appears to be independent of the intensity of the primary infection.

Passive transfer of immune serum to naïve fish conferred partial protection against *T. danilewskyi*. At a first glance, this finding appears to contradict the results obtained in the immunization experiments (i.e., all immunized groups with a significantly enhanced antibody response (0 dpi) had parasitemias that were significantly greater than the control fish). The specificity of the humoral response may account for this discrepancy. That is to say, the antibody specificity of serum from a recovered fish may be different from that in a fish immunized with parasite extract.

Of the five parameters examined in the active immunization experiments, the prevalence of infection and abundance of parasites were the best indicators of conferred protection. Because these processes were cumbersome and limited to the examination of a small volume of blood, it would be beneficial to re-evaluate the methods that are presently being used in this area of research. For example, abundance of fish kinetoplastids was commonly determined by microscopic examination of wet mount preparations (i.e., haemocytometer, Bürker counting chamber, or the haematocrit centrifuge technique). My attempts to automate this procedure, using flow cytometry, were met with limited success. Based on cell size and internal complexity, the flow cytometer was unable to distinguish leukocytes from trypanosomes. To successfully automate this process, a third discriminating parameter would be required. For example, production and application of a fluorescent-labeled rabbit anti-*T. danilewskyi* antibody would facilitate the identification of trypanosomes by the flow cytometer. Other possible methods include the use of automated counters (i.e., Coulter Counter, Coulter Corp., Hialeah, FL) or cellular assays that measure the reduction of tetrazolium salts [34, 219, 231].

In fish parasite systems, immense variability between cohorts is commonly a hindrance to workers conducting experiments *in vivo*. Because numerous inherent factors (i.e., genetic variation, social behavior, and gender) may contribute to this variability, it was unlikely that increasing the sample size, by a reasonable amount, would resolve this issue. Given these circumstances, it would be useful to define individual fish in an outbred population as a high or low responder. One such study determined the susceptibility of male and female goldfish. Wang (1994) determined that the dynamics of *T. danilewskyi* infection in male goldfish was similar to that in females; however, the males were slightly more susceptible [283]. Another study illustrated that carp highly susceptible to *T. borreli* have a genetically predetermined low antibody response [291].

Mortality was a poor indicator of the efficacy of immunization. Likewise, red cell volume was an unreliable predictor of resistance. Specifically, red cell volumes in fish, immunized with ES products and FCA, were notably lower than in control fish with higher parasitemia. Further, red cell volumes may be sensitive to experimental procedures.

Detection of antibody by the ELISA was a poor indicator of the efficacy of the immunization procedure. Further, the role of parasite-specific antibodies in eliminating *T. danilewskyi* is ill defined. In several instances, my results showed a positive correlation between parasitemia and the antibody response. As discussed previously (Chapter V), it seems that the effectiveness of antibodies was contingent on the phase of the infection. To clarify these concerns, it would be advantageous to study the time intervals and dosage of immune IgM in a passive transfer experiment.

Active immunization with ES products suspended in FCA increased the resistance of goldfish to *T. danilewskyi*. This protection was reflected in the prevalence of infection and the abundance of parasites. For the development of a vaccine against fish trypanosomiasis, it would be beneficial for future studies to isolate and characterize the molecule(s) that elicit protection. Further, it is imperative to optimize the conditions of immunization. For instance, it is important to determine if immunization with ES products, suspended in FCA, could be administered by a method that is feasible for application by a commercial fishery. It is also necessary to determine how long the protection is conferred after immunization and whether booster injections would be required.

Investigation of the cell-mediated response to parasite-released molecules is warranted, as significant levels of protection were conferred by administration of ES products with FCA. As stated earlier, our knowledge of cell-mediated immune responses in protozoan infection of fish is in its infancy. Likewise, our knowledge of cell-mediated responses in fish is limited, as few cell surface markers have been defined and virtually no reagents are commercially available. Notwithstanding, isolation of T-like cells should be possible through negative selection procedures. The resulting cell populations could then be used in studies of blastogenic responses, adoptive transfer, macrophage inhibition assays, etc.

Immunization with sodium perchlorate-treated trypanosomes (intravenously) or detergent-soluble extracts (intramuscularly or intraperitoneally) merits further investigation, as the abundance of parasites in these groups were notably lower than in the respective control group. Presumably, these extracts represent the surface antigens that were accessible to the immune system in a primary infection. Consequently, it seems reasonable to anticipate antigenic similarities between these two extracts. Another interesting aspect would be to determine if the immunogenic molecules of ES products represent aggregations of surface bound antigens.

Protection against *T. danilewskyi*, immunization with parasite lysate or water-soluble molecules was futile. Nevertheless, results from these experiments address aspects of the fish-trypanosome relationship that have not been presented in the literature. As illustrated in mammalian trypanosome systems, it was possible that parasite molecules (irrelevant to elimination of the infection) served as a blastogenic stimulus for B-cells [17, 18]. This process would result in general immunosuppression that could be attributed to polyclonal activation and subsequent clonal exhaustion of B-cells. Furthermore, similar to that reported in mammalian trypanosomes, useful information may emerge from studies that determine the function of *T. danilewskyi* molecules in the induction of tolerance, antigenic competition, and clonal expansion of suppressor-like cells.

Administration route (i.e., intravenous, intramuscular, or intraperitoneal) of active immunization does not appear to influence the outcome of the challenge infection. Evidently, the discriminating factors were the antigen preparation and the vehicle.

Administration of control solution conferred significant levels of protection. This conclusion was drawn from comparisons between groups that were inoculated with control solution (with or without adjuvant) and those fish that were not immunized. Other studies have attributed such observations to the elicitation of a nonspecific response. [114, 210]. Application of this information may allow protection against *T. danilewskyi* over a short time period.

In closing, results obtained from this research augmented our knowledge about the biology of the host-parasite association, including identification of components that influence susceptibility of fish, antigenic analyses of trypanosomes, and the *in vitro* growth requirements of *T. danilewskyi*. Furthermore, results from my experiments have necessitated the re-examination of inferences that have been drawn from the sparse amount of literature that is currently available. As a result, I believe these findings will serve as a first step toward developing vaccination strategies or immunomodulation therapies for use in aquaculture facilities with outbreaks of trypanosomiasis.

LITERATURE CITED

1. Abdallah, M.A., Abdel-Hafez, S.K., and Al-Yaman, F.M. 1990. *Trypanosoma acomys* (Wenyon, 1909): Continuous culturing with a mouse fibroblast cell-line (A9). Journal of Protozoology, 37:113-117.

2. Ada, G.L., 1993. Vaccines. In Fundamental Immunology, eds Paul, W.E. Raven Press Ltd., New York, 1993, p. 1309-1352.

3. Adroher, F.J., Lupianez, J.A., and Osuna, A. 1988. Influence of saccharides and sodium chloride on growth and differentiation of *Trypanosoma cruzi*. Cell Differentiation, 22:165-170.

4. Akadmiia nauk, S.S.S.R., Bykovskaia, P., and Pavloskii, E.N. 1964. Key to parasites of freshwater fish of the U.S.S.R. Vol. 3. Israel Program for Scientific Translations Ltd., Jerusalem.

5. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1989. Molecular Biology of the Cell. 2nd ed. Garland Publishing Inc., New York and London.

6. Albesa, I. and Eraso, A.J. 1983. Primary isolation of *Trypanosoma cruzi* by hemoculture influence of glucose concentration. American Journal of Tropical Medicine & Hygiene, 32:963-967.

7. Albright, J.W. and Albright, J.F. 1978. Growth of *Trypanosoma musculi* in cultures of murine spleen cells and analysis of the requirement for supportive spleen cells. Infection & Immunity, 22:343-349.

8. Albright, J.W. and Albright, J.F. 1980. *In vitro* growth of *Trypanosoma musculi* in cell-free medium conditioned by rodent macrophages and mercaptoethanol. International Journal for Parasitology, 10:137-142.

9. Albright, J.W. and Albright, J.F. 1981. Basis of specificity of rodent trypanosomes for their natural hosts. Infection & Immunity, 33:355-363.

10. Albright, J.W. and Albright, J.F. 1981. Differences in reisitance to *Trypanosoma musculi* infection among strains of inbred mice. Infection & Immunity, 33:364-371.

11. Allison, A.C. and Byars, N.E. 1991. Immunological adjuvants: Desirable properties and side-effects. Molecular Immunology, 28:279-284.

12. Ardelli, B.F., Forward, G.M., and Woo, P.T.K. 1994. Brook charr, *Salvelinus fontinalis* (Mitchill), and cryptobiosis: A potential salmonid reservoir host for *Cryptobia salmositica* Katz, 1951. Journal of Fish Diseases, 17:567-577.

13. Ardelli, B.F. and Woo, P.T.K. 1997. Protective antibodies and anamnestic response in *Salvelinus fontinalis* to *Cryptobia salmositica* and innate resistance of *Salvelinus namaycush* to the hemoflagellate. Journal of Parasitology, 83:943-946.

14. Ardelli, B.F. and Woo, P.T.K. 1998. Improved culture media for piscine hemoflagellates, *Cryptobia* and *Trypanosoma* (Kinetoplastida). Journal of Parasitology, 84:1267-1271.

15. Ardelli, B.F. and Woo, P.T.K. 1999. The therapeutic use of isometamidium chloride against *Cryptobia salmositica* in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms, 37:195-203.

16. Arevalo, J., Panebra, A., and Cruz, C.S. 1985. Relavance of hemin for *in vitro* differentiation of *Trypanosoma cruzi*. Journal of Protozoology, 32:553-555.

17. Assoku, R.K.G., Hazlett, C.A., and Tizard, I. 1979. Immunosuppression in experimental African trypanosomiasis. Polyclonal B-cell activation and mitogenicity of trypanosome-derived saturated fatty acids. International Archives of Allergy & Applied Immunology, 59:298-307.

18. Assoku, R.K.G., Tizard, I.R., and Nielsen, K.H. 1977. Free fatty acids, complement activation, and polyclonal B-cell stimulation as factors in the immunopathogenesis of African trypanosomiasis. Lancet, 2:956-959.

19. Avila, J.L., Bretana, A., Casanova, M.A., Avila, A., and Rodriguez, F. 1979. *Trypanosoma cruzi*: Defined medium for continous cultivation of virulent parasites. Experimental Parasitology, 48:27-35.

20. Baker, J.R. and Selden, L.F. 1978. *Trypanosoma* (*Scizotrypanum*) *dionisii*: Influence of mouse peritoneal macrophages and calf sera on extracellular growth *in vitro* at 37C. Journal of General Microbiology, 106:27-32.

21. Balber, A.E. 1983. Primary murine bone marrow cultures support continuous growth of infectious human trypanosomes. Science, 220:421-423.

22. Balber, A.E. 1984. Growth of *Trypanosoma brucei gambiense, T. b. rhodesiense*, and *T. congolense* in bone marrow cultures of genetically anemic Sl/Sld mice. Journal of Parasitology, 70:179-180.

23. Balber, A.E., Gonias, S.L., and Pizzo, S.V. 1985. *Trypanosoma brucei gambiense*: Growth *in vitro* of bloodstream forms inhibited by dichlorodiammineplatinum (II) compounds and hypolipidemic drugs. Experimental Parasitology, 59:74-80.

24. Baltz, T., Baltz, D., Giruod, C., and Crockett, J. 1985. Cultivation of semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodensiense, and T. gambiense*. Embo Journal, 4:1273-1277.

25. Barahona-Fernandes, M.H. and Dinis, M.T. 1992. Leech control (*Platybdella soleae*, Hirudinae) on flatfishes in an aquaculture unit. Publicacoes do Instituto de Zoologia, 226:1-4.

26. Barr, S.C., Bowman, D.D., Phillips, L.G., and Barr, M.C. 1993. *Trypanosoma manulis* N. Sp. from the Russian pallas cat *Felis manul*. Journal of Eukaryotic Microbiology, 40:233-237.

27. Barrow, J.H. 1954. Observations of some host-specificity and immunological reactions of trypanosome infections in some freshwater fish of Europe. Anatomical Record, 120:750-751.

28. Barrow, J.H. 1955. Social behavior in fresh-water fish and its effect on resistance to trypanosomes. Proceedings of the National Academy of Sciences USA, 41:676-679.

29. Beamish, F.W.H., Sitja-Bobadilla, A., Jebbink, J.A., and Woo, P.T.K. 1996. Bioenergetic cost of cryptobiosis in fish: Rainbow trout *Oncorhynchus mykiss* infected with *Cryptobia salmositica* and with an attenuated live vaccine. Diseases of Aquatic Organisms, 25:1-8.

30. Becker, C.D., 1977. Flagellate parasites of fish. In Parasitic Protozoa, eds Kreier, J.P. Academic Press, New York, 1977, p. 357-416.

31. Behr, M.A., Mathews, S.A., and D'Alesandro, P.A. 1990. A medium for the continuous cultivation of bloodstream forms of *Trypanosoma lewisi* at 37 C. Journal of Parasitology, 76:711-716.

32. Berens, R.L., Marr, J.J., LaFon, S.W., and Nelson, D.J. 1981. Purine metabolism in *Trypanosoma cruzi*. Molecular & Biochemical Parasitology, 3:187-196.

33. Bernardet, J.F. 1997. Immunization with bacterial antigens: *Flavobacterium* and *Flexibacter* infection. Developments in Biological Standardization, 90:179-188.

34. Berridge, M.V., Tan, A.S., McCoy, K., and Wang, R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. Biochemica, 4:14-19.

35. Bienek, D.R. and Belosevic, M. 1997. Comparative assessment of growth of *Trypanosoma danilewskyi* (Laveran & Mesnil) in medium containing fish or mammalian serum. Journal of Fish Diseases, 20:217-221.

36. Bienek, D.R. and Belosevic, M. 1999. Macrophage or fibroblast-conditioned medium potentiates growth of *Trypanosoma danilewskyi* Lavern & Mesnil, 1904. Journal of Fish Diseases, 22:1-9.

37. Black, S. and Vandeweerd, V. 1989. Serum lipoproteins are required for multiplication of *Trypanosoma brucei brucei* under axenic culture conditions. Molecular & Biochemical Parasitology, 37:65-72.

38. Bly, J.E., Grimm, A.S., and Morris, I.G. 1986. Transfer of passive immunity from mother to young in a teleost fish: Haemagglutininating activity in the serum and eggs of plaice, *Pleuronectes platessa* L. Comparative Biochemistry & Physiology, 84A:309-313.

39. Bogitsh, B.J., Ribeiro-Rodrigues, R., and Carter, C.E. 1995. *In vitro* effects of mannan an Cytochalasin B on the uptake of horseradish peroxidase and [14C] sucrose by *Trypanosoma cruzi* epimastigotes. Journal of Parasitology, 81:144-148.

40. Bogner, K.H. and Ellis, A.E. 1976. Properties and functions of lymphocytes and lymphoid tissues in teleost fish. in Munich Seminar on Biology of Fish. Munich: Gustav Fischer Verlag.

41. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 Solution. Journal of Biological Chemistry, 256:1604-1607.

42. Bower, S.M. and Evelyn, T.P.T. 1988. Acquired and innate resistance to the haemoflagellate *Cryptobia salmositica* in sockeye salmon (*Onchorhynchus nerka*). Developmental & Comparative Immunology, 12:749-760.

43. Bower, S.M. and Margolis, L. 1984. Detection of infection and susceptibility of different Pacific salmon stocks (*Oncorhynchus* spp.) to the haemoflagellate *Cryptobia salmositica*. Journal of Parasitology, 70:273-278.

44. Bower, S.M. and Woo, P.T.K. 1977. *Cryptobia catostomi*: Incubation in plasma of susceptible and refractory fishes. Experimental Parasitology, 43:63-68.

45. Brown, K.N., Armstrong, J.A., and Valentine, R.C. 1965. The ingestion of protein molecules by blood forms of *Trypanosoma rhodesiense*. Experimental Cell Research, 39:129-135.

46. Brun, R. 1982. Cultivation of procyclic trypomastigotes of *Trypanosoma congolense* in a semi-defined medium with direct adaptation from bloodstream forms. Zeitschrift fur Parasitenkunde, 67:129-135.

47. Brun, R., Grootenhuis, J.G., Kunz, C., and Schonenberger, M. 1984. *In vitro* cultivation of *Trypanosoma* (*T.*) *brucei* bloodstream forms using cell lines and sera from African wild bovidae. Journal of Parasitology, 70:836-837.

48. Brun, R. and Jenni, L. 1977. A new semi-defined medium for *Trypanosoma brucei* spp. Acta Tropica, 34:21-33.

49. Brun, R., Schonenberger, J.M., and Schell, K.F. 1981. *In vitro* cultivation of bloodstream forms of *Trypanosoma brucei, T. rhodesiense*, and *T. gambiense*. Journal of Protozoology, 28:470-479.

50. Buchmann, K., Lindenstrom, T., and Sigh, J. 1999. Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjavini* immunized rainbow trout. Journal of Helminthology, 73:189-195.

51. Burreson, E.M. 1982. Trypanoplasmosis in flounder along the Atlantic coast of the United States. in Antigens of fish pathogens: Development and production for vaccines and serodiagnostics. Collection Fondation Marcel Merieux.

52. Burreson, E.M. and Frizzell, L.J. 1986. The seasonal antibody response in juvenile summer flounder (*Paralichthys dentatus*) to the hemoflagellate *Trypanoplasma bullocki*. Veterinary Immunology & Immunopathology, 12:395-402.

53. Caceres, O. and Fernandes, J.F. 1976. Glucose metabolism, growth and differentiation of *Trypanosoma cruzi*. Revista Brasileira de Biologia, 36:397-410.

54. Carrington, M., 1993. Culturing and biological cloning of *Trypanosoma brucei*. In Methods in Molecular Biology, eds Hyde, J.E. Humana Press Inc., Totowa, NJ, 1993, p. 1-13.

55. Cazzulo, J.J., Franke de Cazzulo, B.M., Engel, J.C., and Cannata, J.J.B. 1985. End products and enzyme levels of aerobic glucose fermentation in trypansomatids. Molecular & Biochemical Parasitology, 16:329-343.

56. Cerban, F., Gruppi, A., Gea, S., and Vottero-Cima, E. 1991. IgG isotype profiles induced in mice by two *Trypanosoma cruzi* electronegative antigen. International Archives of Allergy & Applied Immunology, 96:35-40.

57. Ceron, C.R., De Araujo Caldas, R., Felix, C.R., Mundim, M.H., and Roitman, I. 1979. Purine metabolism in trypanosomatids. Journal of Protozoology, 26:479-483.

58. Chilmonczyk, S., 1992. The thymus in fish: development and possible function in the immune response. In Annual Review of Fish Diseases, eds Faisal, M. and Hetrick, F.M. Pergamon Press, New York, 1992, p. 181-200.

59. Clark, T.G., Dickerson, H.W., Gratzek, J.B., and Findly, R.C. 1987. *In vitro* response of *Ichthyophthirius multifiliis* to sera from immune channel catfish. Journal of Fish Biology, 31 (Supplement A):203-208.

60. Clark, T.G., Lin, T.L., and Dickerson, H.W. 1996. Surface antigen cross-linking triggers forced exit of a protozoan parasite from its host. Proceedings of the National Academy of Sciences, USA, 93:6825-6829.

61. Coppens, I., Levade, T., and Courtoy, P.J. 1995. Host plasma low density lipoprotein particles as an essential source of lipids for the bloodstream forms of *Trypanosoma brucei*. Journal of Biological Chemistry, 270:5736-5741.

62. Coppens, I., Opperdoes, F.R., Courtoy, P.J., and Baudhuin, P. 1987. Receptor-mediated endocytosis in the bloodstream form of *Trypanosoma brucei*. Journal of Protozoology, 34:465-473.

63. Cottrell, B.J. 1977. A trypanosome from the plaice, *Pleuronectes platessa* L. Journal of Fish Biology, 11:35-47.

64. Cross, G.A.M., Klein, R.A., and Linstead, D.J. 1975. Utilization of amino acids by *Trypanosoma brucei* in culture: L-threonine as a precursor for acetate. Parasitology, 71:311-326.

65. Czichos, J., Nonnengaesser, C., and Overath, P. 1986. *Trypanosoma brucei*: *cis*-Aconitate and temperature reduction as triggers of synchronous transformation of bloodstream to procyclic trypomastigotes *in vitro*. Experimental Parasitology, 62:283-291.

66. Davies, A.J., Mastri, C., Ariyanayagam, M.R., Sappal, G.K., and Wilkins, T.A. 1992. Simple methods for culturing and preserving *Trypanosoma granulosum* Laveran and Mesnil from European eels (*Anguilla anguilla* L.). Bulletin of the European Association of Fish Pathologists, 12:8-10.

67. Davies, A.J., Mastri, C., Thorborn, D.E., and Mackintosh, D. 1995. Experiments with growth of the eel trypanosome, *Trypanosoma granulosum* Laveran & Mesnil, 1902, in semi-defined and defined media. Journal of Fish Diseases, 18:599-608.

68. Davies, M.J., Ross, A.M., and Gutteridge, W.E. 1983. The enzymes of purine salvage in *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania mexicana*. Parasitology, 87:211-217.

69. De Souza, W. and Meyer, H. 1975. An electron microscopic and cytochemical study of the cell coat of *Trypanosoma cruzi* in tissue cultures. Zeitscrift fur Parasitenkunde, 46:179-187.

70. DeLuna, R., *et al.* 1999. Early suppression of lymphoproliferative response in dogs with natural infections by *Leishmania infantum*. Veterinary Immunology & Immunopathology, 70:95-103.

71. Dickerson, H.W., Brown, J., Dawe, D.L., and Gratzek, J.B. 1984. *Tetrahymena pyriformis* as a protective antigen against *Ichthyophthirius multifiliis* infection: Comparisons between isolates and ciliary preparations. Journal of Fish Biology, 24:523-528.

72. Dickerson, H.W., Clark, T.G., and Leff, A.A. 1993. Serotypic variation among isolates of *Ichthyophthirius multifiliis* based on immobilization. Journal of Eukaryotic Microbiology, 40:816-820.

73. Dogiel, V.A., Petrushevski, G.K., and Polyanski, Y.I., 1961. ed. Parasitology of fishes. Oliver & Boyd Ltd.: Edinburgh & London. 384.

74. Durum, S.K. and Oppenheim, J.J., 1993. Proinflammatory cytokines and immunity. In Fundamental Immunology, eds Paul, W.E. Raven Press, New York, 1993, p. 801-835.

75. Dusanic, D.G. 1968. Growth and immunologic studies on the culture forms of *Trypanosoma lewisi*. Journal of Protozoology, 15:328-333.

76. Dusanic, D.G. 1969. Cultivation of *Trypanosoma lewisi* in a dialysate medium-I. Amino acid alterations during growth. Comparative Biochemistry & Physiology, 30:895-901.

77. Duszenko, M., Ferguson, M.A.J., Lamont, G.S., Rifkin, M.R., and Cross, G.A.M. 1985. Cysteine eliminates the feeder cell requirement for cultivation of *Trypanosoma brucei* bloodstream forms *in vitro*. Journal of Experimental Medicine, 162:1256-1263.

78. Duszenko, M., Muhlstadt, K., and Broder, A. 1992. Cysteine is an essential growth factor for *Trypanosoma brucei* bloodstream forms. Molecular & Biochemical Parasitology, 50:269-274.

79. Dvorak, J.A., Engel, J.C., Leapman, R.D., Swyt, C.R., and Pella, P.A. 1988. *Trypanosoma cruzi*: Elemental compostion heterogeneity of cloned stocks. Molecular & Biochemical Parsitology, 31:19-26.

80. Dykova, I. and Lom, J. 1978. Tissue reaction of the three-spined stickleback *Gasterosteus aculeatus* L. to infection with *Glugea anomala* (Moniez, 1887). Journal of Fish Diseases, 1:83-90.

81. Dykova, I. and Lom, J. 1979. Histopathological changes in *Trypanosoma danilewskyi* Laveran & Mesnil, 1904 and *Trypanoplasma borelli* Laveran & Mesnil, 1902 infections of goldfish, *Carassius auratus* (L.). Journal of Fish Diseases, 2:381-390.

82. Dykova, I., Lom, J., and Egusa, S. 1980. Tissue reaction to *Glugea plecoglossi* infection in its natural host, *Plecoglossus altivelis*. Folia Parasitologica, 27:213-216.

83. El-On, J. and Greenblatt, C.L. 1977. *Trypanosoma lewisi*, *T. acomys*, and *T. cruzi*: A method for their cultivation with mammalian tissue. Experimental Parasitology, 41:31-42.

84. Evans, D.L., Carlson, R.L., Graves, S.S., and Hogan, K.T. 1984. Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) IV. Target cell binding and recycling capacity. Developmental & Comparative Immunology, 8:823-833.

85. Evans, D.L., Graves, S.S., Cobb, D., and Dawe, D.L. 1984. Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) II. Parameters of target cell lysis and specificity. Developmental & Comparative Immunology, 8:303-312.

86. Evans, D.L., Hogan, K.T., Graves, S.S., Carlson, R.L., Floyd, E., and Dawe, D.L. 1984. Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) III. Biophysical and biochemical properties affecting cytolysis. Developmental & Comparative Immunology, 8:599-610.

87. Evans, D.L., Jaso-Friedmann, L., Smith, E.E., St. John, A., Koren, H.S., and Harris, D.T. 1988. Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies. Journal of Immunology, 141:324-332.

88. Evans, D.L., Leary, J.H., Nadella, P., and Jaso-Friedmann, L. 1998. Evidence for antigen recognition by nonspecific cytotoxic cells: Initiation of 3H-thymidine uptake following stimulation by a protozoan parasite and homologous cognate synthetic peptide. Developmental & Comparative Immunology, 22:161-172.

89. Evans, D.L., Leary, J.H., Weisman, Z., Warren, J., and Jaso-Friedmann. 1996. Mapping of the epitope recognized by non-specific cytotoxic cells: Determination of the fine specificity using synthetic peptides. Scandinavian Journal of Immunology, 43:556-565.

90. Evans, D.L., Smith, E.E., and Brown, F.E. 1987. Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) VI. Flow cytometric analysis. Developmental & Comparative Immunology, 11:95-104.

91. Feng, S. and Woo, P.T.K. 1996. Biological characterization of a monoclonal antibody against a surface membrane antigen on *Cryptobia salmositica* Katz, 1951. Journal of Fish Diseases, 19:137-143.

92. Feng, S. and Woo, P.T.K. 1996. Cell-mediated immune response and T-like cells in thymectomized *Oncorhynchus mykiss* (Walbaum) infected with or vaccinated against the pathogenic haemoflagellate *Cryptobia salmositica* Katz, 1951. Parasitology Research, 82:604-611.

93. Feng, S. and Woo, P.T.K. 1997. Complement fixing antibody production in thymectomized *Oncorhynchus mykiss*, vaccinated against or infected with the pathogenic haemoflagellate *Cryptobia salmositica*. Folia Parasitologica, 44:188-194.

94. Feng, S. and Woo, P.T.K. 1997. Therapeutic and prophylactic effects of a protective monoclonal antibody (MAb-001) against the pathogenic haemoflagellate *Cryptobia salmositica*. Diseases of Aquatic Organisms, 28:211-219.

95. Feng, S. and Woo, P.T.K. 1998. Characterization of a 200 kDa glycoprotein (Cs-gp200) on the pathogenic piscine haemoflagellate *Cryptobia salmositica*. Diseases of Aquatic Organisms, 32:41-48.

96. Ferguson, H. 1996. Health and disease in freshwater fish: An overview. in Health and Disease in Wild Freshwater Fish. Saskatoon, Saskatchewan:

97. Fernandes, J.F., Castellani, O., and Kimura, E. 1969. Physiological events in the course of growth and differentiation of *Trypanosoma cruzi*. Genetic Supplement, 61:213-226.

98. Figueroa, F., Mayer, W.E., Lom, J., Dykova, I., Weller, M., Peckova, H., and Klein, J. 1999. Fish trypanosomes: Their position in kinetoplastid phylogeny and variability as determined from 12S rRNA kinetoplast sequences. Journal of Eukaryotic Microbiology, 46:473-481.

99. Fischer, E., Ouaissi, M.A., Velge, P., Cornette, J., and Kazatchkine, M.D. 1988. gp 58/68, a parasite component that contributes to the escape of the trypomastigote form of *T. cruzi* from damage by the human alternative complement pathway. Immunology, 65:299-303.

100. Fish, W.R., Marr, J.J., and Berens, R.L. 1982. Purine metabolism in *Trypanosoma brucei gambiense*. Biochimica et Biophysica Acta, 714:422-428.

101. Forward, G.M., Ferguson, M.M., and Woo, P.T.K. 1995. Susceptibility of brook charr, *Salvelinus fontinalis* to the pathogenic haemoflagellate, *Cryptobia salmositica*, and the inheritance of innate resistance by progenies of resistant fish. Parasitology, 111:337-345.

102. Forward, G.M. and Woo, P.T.K. 1996. An *in vitro* study on the mechanism of innate immunity in *Cryptobia*-resistant brook charr (*Salvelinus fontinalis*) against *Cryptobia salmositica*. Parasitology Research, 82:238-241.

103. Gallin, J.I., 1993. Inflammation. In Fundamental Immunology, eds Paul, W.E. Raven Press, New York, 1993, p. 1015-1032.

104. Garcia, E.S., Gonzalez, M.S., De Azambuja, P., Baralle, F.E., Fraidenraich, D., Torres, H.N., and Flawia, M.M. 1995. Induction of *Trypanosoma cruzi* metacyclogenesis in the gut of a hematophagous insect vector, *Rhodnius prolixus*, by hemoglobin and peptides carrying alphaD-Globin Sequences. Experimental Parasitology, 81:255-261.

105. Gebremedhin, G., Adewunmi, C.O., Becker, W., Agbedahunsi, J.M., and Dorfler, G. 1994. Hirudinicidal activities of some natural molluscicides used in schistosomiasis control. Journal of Ethnopharmacology, 41:127-132.

106. Geigy, R., Steiger, R., and Hecker, H. 1970. Beitrage zur pinocytose von *Trypanosoma* (Trypanozoon) *brucei*, Plimmer & Bradfort, 1899. Acta Tropica, 27:271-277.

107. Golub, E.S. and Green, D.R. 1991. Immunology A Synthesis. 2nd ed. Sinauer Associates Inc., Sunderland, Massachusetts.

108. Gonclaves, M.F., Umezawa, E.S., Katzin, A.M., deSouza, W., Alves, M.J.M., Zingales, B., and Colli, W. 1991. *Trypanosoma cruzi*: Shedding of surface antigens as membrane vesicles. Experimental Parasitology, 72:43-53.

109. Goven, B.A., Dawe, D.L., and Gratzek, J.B. 1980. Protection of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* Fouquet by immunization. Journal of Fish Biology, 17:311-316.

110. Goven, B.A., Dawe, D.L., and Gratzek, J.B. 1981. Protection of channel catfish (*Ictalurus punctatus*) against *Ichthyophthirius multifiliis* (Fouquet) by immunization with varying doses of *Tetrahymena pyriformis* (Lwoff) cilia. Aquaculture, 23:269-273.

111. Grab, D.J., Shaw, M.K., Wells, C.W., Verjee, Y., Russo, D.C.W., Webster, P., Naessens, J., and Fish, W.R. 1993. The transferrin receptor in African trypanosomes: Identification, partial characterization and subcellular localization. European Journal of Cell Biology, 62:114-126.

112. Graves, S.S., Evans, D.L., and Dawe, D.L. 1985. Antiprotozoan activity of nonspecific cytotoxic cells (NCC) from the channel catfish (*Ictalurus punctatus*). Journal of Immunology, 134:78-85.

113. Graves, S.S., Evans, D.L., and Dawe, D.L. 1985. Mobilization and activation of nonspecific cytotoxic cells (NCC) in the channel catfish (*Ictalurus punctatus*) infected with *Ichthyophthirius multifiliis*. Comparative Immunology Microbiology & Infectious Disease, 8:43-51.

114. Grayson, T.H., Williams, R.J., Wrathmell, A.B., Munn, C.B., and Harris, J.E. 1987. Effects of immunopotentiating agents on the immune response or rainbow trout, *Salmo gairdneri* Richardson, to ERM vaccine. Journal of Fish Biology, 31:195-202.

115. Greenblatt, C.L., Jori, L.A., and Cohnmann, H.J. 1969. Chromatographic separation of a rat serum growth factor required by *Trypanosoma lewisi*. Experimental Parasitology, 24:228-242.

116. Greenblatt, C.L. and Tyroler, E. 1971. *Trypanosoma lewisi*: *In vitro* behavior of rat spleen cells. Experimental Parasitology, 30:363-374.

117. Greenblatt, H.C., Diggs, C.L., and Aikawa, M. 1983. Antibody-dependent phagocytosis of *Trypanosoma rhodesiense* by murine macrophages. American Journal of Tropical Medicine & Hygiene, 32:34-45.

118. Gruenberg, J., Sharma, P.R., and Deshusses, J. 1978. D-Glucose Transport in *Trypanosoma brucei* D-Glucose transport is the rate limiting step of its metabolism. European Journal of Biochemistry, 89:461-469.

119. Gruppi, A., Pistoresi-Palencia, M.C., Basso, B., and Vottero-Cima, E. 1990. Mouse IgE response a exoantigens of *Trypanosoma cruzi*. International Archives of Allergy & Applied Immunology, 92:56-59.

120. Guimaraes, R.C. and Gutteridge, W.E. 1986. Uptake of purine bases by *Trypanosoma cruzi* culture epimastigotes. Brazilian Journal of Medical & Biological Research, 19:339-350.

121. Guimaraes, R.C. and Gutteridge, W.E. 1987. Purine base uptake in *Trypanosoma cruzi*:Adaptations and effects of inhibitors. Brazilian Journal of Medical & Biological Research, 20:1-10.

122. Gutteridge, W.E. and Davies, M.J. 1981. Enzymes of purine salvage in *Trypanosoma cruzi*. FEBS Letters, 127:211-214.

123. Guy, R.A.Mechanisms of entry of amastigotes of *Leishmania major* into macrophages *Department of Biological Sciences*. 1995, University of Alberta: Edmonton. p. 248.

124. Hammond, D.J. and Gutteridge, W.E. 1984. Purine and pyrimidine metabolism in the trypanosomatidae. Molecular & Biochemical Parasitology, 13:243-261.

125. Havelka, J., Volf, F., and Tesarcik, J. 1965. Investigation of antiparasitic and antimycotic measures I. Investigation of new endoparasitica with special regard to *Cryptobia cyprini* (Plehn, 1903) (syn.: *Trypanoplasma cyprini*, 1903). Prace Vurh Vodnany, 5:68-87.

126. Henley, G.L., Lee, C.M., and Takeuchi, A. 1978. Electron microscopy observations on *Trypanosoma brucei*: Freeze-cleaving and thin-sectioning of the apical part of the flagellar pocket. Zeitschrift fur Parasitenkunde, 55:181-187.

127. Herwig, N. 1979. Handbook of Drugs and Chemicals used in the Treatment of Fish Deseases. Charles C. Thomas Publisher, Springfield.

128. Hide, G., Gray, A., Harrison, C.M., and Tait, A. 1989. Identification of epidermal growth factor receptor homologue in trypanosomes. Molecular & Biochemical Parasitology, 36:51-60.

129. Hide, G., Gray, A., Harrison, C.M., and Tait, A. 1990. Identification of an EGF homologue in trypanosomes. Parasites: Molecular Biology, Drug and Vaccine Design, :213-224.

130. Hill, G.C., Shimer, S., Caughey, B., and Sauer, S. 1978. Growth of infective forms of *Trypanosoma (T.) brucei* on buffalo lung and Chinese hamster lung tissue culture cells. Acta Tropica, 35:201-207.

131. Hill, G.C., Shimer, S.P., Caughey, B., and Sauer, L.S. 1978. Growth of infective forms of *Trypanosoma rhodesiense in vitro* the causitive agent of African trypanosomiasis. Science, 202:763-765.

132. Hines, R.S. and Spira, D.T. 1974. Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.) V. Acquired immunity. Journal of Fish Biology, 6:373-378.

133. Hirumi, H., Doyle, J.J., and Hirumi, K. 1977. African trypanosomes: Cultivation of animal-infective *Trypanosoma brucei in vitro*. Science, 196:992-994.

134. Horne, M.T., Roberts, R.J., and Tatner, M. 1984. The effects of the use of potassium alum adjuvant in vaccines against vibriosis in rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Diseases, 7:91-99.

135. Hsu, J.P., Huang, S.T., Peng, T.F., and Hung, H.H. 1995. Case report: Trypanosomiasis of cultured freshwater fish (*Cichlasoma managuense*). Journal of the Chinese Society of Veterinary Science, 21:111-116.

136. Ingram, G.A. and Alexander, J.B. 1976. The immune response of brown trout (*Salmo trutta* L.) to injection with soluble antigens. Acta Biologica Medica Germanica, 35:1561-1570.

137. Isakov, N., Tamir, A., and El-On, J. 1994. Suppression of antigen-specific T cell responses by *Leishmania major* excreted factor: Inhibition of activation signals linked to the T cell antigen receptor and interleukin 2 receptor. Israel Journal of Medical Sciences, 30:673-679.

138. Isalm, A.K.M.N. and Woo, P.T.K. 1991. Anorexia in goldfish *Carassius auratus* infected with *Trypanosoma danilewskyi*. Diseases of Aquatic Organisms, 11:45-48.

139. Islam, A.K.M.N. and Woo, P.T.K. 1991. Anemia and its mechanisms in goldfish *Carassius auratus* infected with *Trypanosoma danilewskyi*. Diseases of Aquatic Organisms, 11:37-43.

140. Islam, A.K.M.N. and Woo, P.T.K. 1991. *Trypanosoma danilewskyi* in *Carassius auratus*: The nature of protective immunity in recovered goldfish. Journal of Parasitology, 77:258-262.

141. Islam, A.K.M.N. and Woo, P.T.K. 1992. Effects of temperature on the *in vivo* and *in vitro* multiplication of *Trypanosoma danilewskyi* Laveran et Mesnil. Folia Parasitologica, 39:1-12.

142. Jaso-Friedmann, L., Leary, J.H., Warren, J., McGraw, R.A., and Evans, D.L. 1997. Molecular characterization of a protozoan parasite target antigen recognized by nonspecific cytotoxic cells. Cellular Immunology, 176:93-102.

143. Jazin, E.E., Luquetti, A.O., Rassi, A., and Frasch., A.C.C. 1991. Shift of excretory-secretory immunogens of *Trypanosoma cruzi* during human Chagas' disease. Infection & Immunity, 59:2189-2191.

144. Jenni, L., *et al.* 1986. Hybrid formation between African trypanosomes during cyclical transmission. Nature, 322:173-175.

145. Joiner, K., Rimoldi, M.T., Kipnis, T., Dias sa Silva, W., Hammer, C.H., and Sher, A. 1987. Trypomastigotes of *Trypanosoma cruzi* produce molecules which accelerate the decay of complement C3 convertases. Complement, 4:175.

146. Joiner, K.A., Dias daSilva, W., Rimoldi, M.T., Hammer, C.H., Sher, A., and Kipnis, T.L. 1988. Biochemical characterization of a factor produced by trypomastigotes of *Trypanosoma cruzi* that accelerates the decay of complement C3 convertase. Journal of Biological Chemistry, 263:11327-11335.

147. Jones, S.R.M., Palmen, M., and van Muiswinkel, W.B. 1993. Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. Veterinary Immunology & Immunopathology, 36:369-378.

148. Jones, S.R.M. and Woo, P.T.K. 1987. The immune response of rainbow trout, *Salmo gairdneri* Richardson, to the haemoflagellate, *Cryptobia salmositica* Katz, 1951. Journal of Fish Diseases, 10:395-402.

149. Jones, S.R.M. and Woo, P.T.K. 1991. Culture characteristics of *Trypanosoma catostomi* and *Trypanosoma phaleri* from North American freshwater fishes. Parasitology, 103:237-243.

150. Jones, S.R.M. and Woo, P.T.K. 1992. Antigenic characterization of cultured trypanosomes isolated from three species of fishes. Systematic Parasitology, 23:43-50.

151. Jones, S.R.M. and Woo, P.T.K. 1993. Biology of *Trypanosoma burresoni* N. SP. from the American eel, *Anguilla rostrata*. Journal of Parasitology, 79:542-547.

152. Jones, S.R.M., Woo, P.T.K., and Stevenson, R.M.W. 1986. Immunosuppression in rainbow trout, *Salmo gairdneri* Richardson, caused by the haemoflagellate *Cryptobia salmositica* Katz, 1951. Journal of Fish Diseases, 9:431-438.

153. Kaattari, S.L., 1992. Fish B lymphocytes: Defining their form and function. In Annual Review of Fish Diseases, eds Faisal, M. and Hetrick, F.M. Pergamon Press, New York, 1992, p. 161-180.

154. Khan, R.A. 1985. Pathogenesis of *Trypanosoma murmanensis* in marine fish of the northwestern Atlantic following experimental transmission. Canadian Journal of Zoology, 63:2141-2144.

155. Khan, R.A. 1988. Experimental transmission, development, and effects of a parasitic copepod, *Lernaeocera branchialis*, on atlantic cod, *Gadus morhua*. Journal of Parasitology, 74:586-599.

156. Kierzenbaum, F. and Budzko, D.B. 1975. Immunization against experimental Chagas' Disease by using culture forms of *Trypanosoma cruzi* killed with a solution of sodium perchlorate. Infection & Immunity, 12:461-465.

157. Killie, J.E.A. and Jorgensen, T.O. 1994. Immunoregulation in fish I. Intermolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (*Salmo salar* L). Developmental & Comparative Immunology, 18:123-136.

158. Killie, J.E.A. and Jorgensen, T.O. 1995. Immunoregulation in fish II. Intermolecular-induced suppression of antibody responses studied by haptenated antigens in Atlantic salmon (*Salmo salar* L). Developmental & Comparative Immunology, 19:389-404.

159. Kim, J.H., Ogawa, K., Takahashi, S., and Wakabayashi, H. 1997. Elevated water temperature treatment of ayu infected with *Glugea plecoglossi*: Apparent lack of involvement of antibody in the effect of the treatment. Fish Pathology, 32:199-204.

160. Kongshavn, P.A. and Ghadirian, E. 1988. Effect of tumor necrosis factor on growth of *Trypanosoma musculi in vivo* and *in vitro*. Advances in Experimental Medicine & Biology, 239:257-262.

161. Kongshavn, P.A.L. and Ghadirian, E. 1988. Enhancing and suppressive effects of tumour necrosis factor/cachectin on growth of *Trypanosoma musculi*. Parasite Immunology, 10:581-588.

162. Koumans-van Diepen, J.C.E., Egberts, E., Peixoto, B.R., Taverne, N., and Rombout, J.H.W.M. 1995. B cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.); an immuno(cyto)chemical study. Developmental & Comparative Immunology, 19:97-108.

163. Kuby, J. 1997. Immunology. 3rd edition. W.H. Freeman & Co., New York.

164. Lalonde, R.G. and Holbein, B.E. 1984. Role of iron in *Trypanosoma cruzi* infection of mice. Journal of Clinical Investigation, 73:470-476.

165. Landsberg, J.H., Smith, S.A., Noga, E.J., and Richards, S.A. 1992. Effect of serum and mucus of blue tilapia, *Oreochromis aureus* on infectivity of the parasitic dinoflagellate, *Amyloodinium* in cell culture. Fish Pathology, 27:163-169.

166. Langreth, S.G. and Balber, A.E. 1975. Protein uptake and digestion in bloodstream and culture forms of *Trypanosoma brucei*. Journal of Protozoology, 22:40-53.

167. Laudan, R., Stolen, J.S., and Cali, A. 1986. The immune response of a marine teleost, *Pseudopleuronectes americanus* (winter flounder) to the protozoan parasite *Glugea stephani*. Veterinary Immunology & Immunopathology, 12:403-412.

168. Laudan, R., Stolen, J.S., and Cali, A. 1986. Immunoglobulin levels of the winter flounder (*Pseudopleuronectes americanus*) and the summer flounder (*Paralichthys dentatus*) injected with the microsporidian parasite *Glugea stephani*. Developmental & Comparative Immunology, 10:331-340.

169. Laudan, R., Stolen, J.S., and Cali, A. 1987. The immunomodulating effect of the microsporidian *Glugea stephani* on the humoral response and immunoglobulin levels in winter flunder, *Pseudopleuronectes americanus*. Journal of Fish Biology, 31 (Supplement A):155-160.

170. Laudan, R., Stolen, J.S., and Cali, A. 1989. The effect of the microsporidia *Glugea stephani* on the immunoglobulin levels of juvenile and adult winter flounder (*Pseudopleuronectes americanus*). Developmental & Comparative Immunology, 13:35-41.

171. Le Page, R.W.F. 1967. Short term cultivation of *Trypanosoma brucei in vitro* at 37 C. Nature, 216:1141-1142.

172. Leary, J.H., Evans, D.L., and Jaso-Friedmann, L. 1994. Partial amino acid sequence of a novel protozoan parasite antigen that inhibits non-specific cytotoxic cell activity. Scandinavian Journal of Immunology, 40:158-164.

173. Leff, A.A., Yoshinaga, T., and Dickerson, H.W. 1994. Cross immunity in channel catfish, *Ictalarus punctatus* (Rafinesque), against two immobilization serotypes of *Ichthyophthirius multifiliis* (Fouquet). Journal of Fish Diseases, 17:429-432.

174. Leiro, J., Ortega, M., Estevez, J., Ubeira, F.M., and Sanmartin, M.L. 1996. The role of opsonization by antibody and complement in *in vitro* phagocytosis of microsporidian parasites by turbot spleen cells. Veterinary Immunology & Immunopathology, 51:201-210.

175. Leiro, J., Ortega, M., Sanmartin, M.L., and Ubeira, F.M. 2000. Non-specific responses of turbot (*Scophthalmus maximus* L.) adherent cells to microsporidian spores. Veterinary Immunology & Immunopathology, 75:81-95.

176. Li, S. and Woo, P.T.K. 1991. Anorexia reduces the severity of cryptobiosis in *Oncorhynchus mykiss*. Journal of Parasitology, 77:467-471.

177. Li, S. and Woo, P.T.K. 1995. Efficacy of a live *Cryptobia salmositica* vaccine, and the mechanism of protection in vaccinated rainbow trout, *Oncorhychus mykiss*, against cryptobiosis. Veterinary Immunology & Immunopathology, 48:343-353.

178. Li, S. and Woo, P.T.K. 1997. Vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against cryptobiosis: Efficacy of the vaccine in fresh and sea water. Journal of Fish Diseases, 20:369-374.

179. Lima, M.F. and Villalta, F. 1990. *Trypanosoma cruzi* receptors for human transferrin and their role. Molecular & Biochemical Parasitology, 38:245-252.

180. Lin, T.L., Clark, T.G., and Dickerson, H. 1996. Passive immunization of channel catfish (*Ictalurus punctatus*) against the ciliated protozoan parasite *Ichthyophthirius multifiliis* by use of murine monoclonal antibodies. Infection & Immunity, 64:4085-4090.

181. Ling, K.H., Sin, Y.M., and Lam, T.J. 1993. Protection of goldfish against some common ectoparasitic protozoans using *Ichthyophthirius multifiliis* and *Tetrahymena pyriformis* for vaccination. Aquaculture, 116:303-314.

182. Lischke, A., Klein, C., Steirhof, Y.D., Hempel, M., Mehlert, A., Almeida, I.C., Ferguson, M.A.J., and Overath, P. 2000. Isolation and characterization of glycosylphosphatidylinositol-anchored mucin-like surface glycoproteins from the bloodstream forms of the freshwater-fish parasite *Trypanosoma carassii*. Biochemical Journal, 345:693-700.

183. Lom, J. 1973. Experimental infection of goldfish with blood flagellates. in International Congress on Protozoology.

184. Lom, J. 1973. Experimental infections of freshwater fishes with blood flagellates. Journal of Protozoology, 20:537.

185. Lom, J., 1979. Biology of fish trypanosomes and trypanoplasms. In Biology of Kinetoplastida, eds Lumsden, W.H.R. and Evans, D.A. Academic Press, New York, 1979, p. 269-337.

186. Lom, J. and Dykova, I. 1992. Protozoan Parasites of Fishes. Vol. 26. Elsevier Science Publishers, Amsterdam.

187. Loo, V.G. and Lalonde, R.G. 1984. Role of iron in intracellular growth of *Trypanosoma cruzi*. Infection & Immunity, 45:726-730.

188. Lu, S. 1975. Culture and morphological observations on *Trypanosoma melophagium*. Chinese Journal of Microbiology, 8:20-35.

189. Marchalonis, J.J. 1971. Isolation and partial characterization of immunoglobulins of goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*). Immunology, 20:161-173.

190. Markevich, A.P. 1963. Parasitic Fauna of Freshwater Fish of the Ukranian S.S.R. Israel Program for Scientific Translations Ltd., Jerusalem.

191. Marr, J.J., 1991. Purine metabolism in parasitic protozoa and its relationship to chemotherapy. In Biochemical Protozoology, eds Coombs, G. and North, M. Taylor & Francis, London, 1991, p. 635.

192. Maslov, D.A., Lukes, J., Jirku, M., and Simpson, L. 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Molecular & Biochemical Parasitology, 75:197-205.

193. McHolland-Raymond, L.E., Kingston, N., and Trueblood, M.S. 1978. Continuous cultivation of *Trypanosoma theileri* at 37 C in bovine cell culture. Journal of Protozoology, 25:388-394.

194. Mehlhorn, H., 1988. ed. Parasitology in Focus Facts and Trends. Springer-Verlag: Berlin. 924.

195. Mendez, Y. and Honigberg, B.M. 1972. Infectivity of *Trypanosoma brucei*-subgroup flagellates maintained in culture. Journal of Parasitology, 58:1122-1136.

196. Meyer, F.P. 1991. Aquaculture disease and health management. Journal of Animal Science, 69:4201-4208.

197. Meyer, H. and De Souza, W. 1973. On the fine structure of *Trypanosoma cruzi* in tissue cultures of pigment epithelium from the chick embryo. Uptake of melanin granules by the parasite. Journal of Protozoology, 20:590-593.

198. Middler, S.A., Kleeman, C.R., and Edwards, E. 1968. Influence of tricaine methane sulfonate anesthesia on fluid and salt metabolism of the toad, *Bufo marinus*. Comparative Biochemistry & Physiology, 24:1065-1067.

199. Midtlyng, P.J. and Lillehaug, A. 1998. Growth of Atlantic salmon *Salmo salar* after intraperitoneal administarion of vaccines containing adjuvants. Diseases of Aquatic Organisms, 32:91-97.

200. Milder, R. and Deane, M.P. 1969. The cytostome of *Trypanosoma cruzi* and *T. conorhini*. Journal of Protozoology, 16:730-737.

201. Mohamed, H.A., Maraghi, S., Wallbanks, K.R., and Molyneux, D.H. 1988. *In vitro* cultivation of *Herpetosoma* trypanosomes on embryonic fibroblasts and in semidefined cell-free medium. Journal of Parasitology, 74:421-426.

202. Mosca, W., Briceno, L., and Hernandez, M.I. 1991. Cell mediated immunity in Chaga's disease. *Trypanosoma cruzi* antigens induce suppression of the *in vitro* proliferative response of mononuclear cells. Memorias do Instituto Oswaldo Cruz., 86:147-152.

203. Neumann, N.F., Barreda, D., and Belosevic, M. 1998. Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Developmental & Comparative Immunology, 22:417-432.

204. Nonaka, M., Yamaguchi, N., Natsuume-Sakai, S., and Takahashi, M. 1981. The complement system of rainbow trout (*Salmo gairdneri*) I. Identification of the serum lytic system homologous to mammalian complement. Journal of Immunology, 126:1489-1494.

205. Nyindo, M., Patel, N., Darji, N., and Golder, T.K. 1979. *Trypanosoma brucei*: *In vitro* propagation of metacyclic forms derived from the salivary glands of *Glossina morsitans*. Journal of Parasitology, 65:751-755.

206. O'Daly, J.A. 1976. Effect of fetal calf serum fractions and proteins on division and transformation of *Trypanosoma* (*Schizotrypanum*) *cruzi in vitro*. Journal of Protozoology, 23:577-583.

207. O'Daly, J.A. and Rodriguez, M.B. 1987. Protein and nucleotide contamination of bovine liver catalase used in culture medium explains growth of *Trypanosoma cruzi*. Transactions of the Royal Society of Tropical Medicine & Hygiene, 81:1-2.

208. O'Daly, J.A., Rodriguez, M.B., and Garlin, G. 1987. *Trypanosoma cruzi*: Growth requirements at different temperatures in fetal bovine serum or peptide supplemented media. Experimental Parasitology, 64:78-87.

209. Ogbunude, P.O.J. and Ikediobi, C.O. 1983. Comparative aspects of purine metabolism in some African trypanosomes. Molecular & Biochemical Parasitology, 9:279-287.

210. Olivier, G., Evelyn, T.P.T., and Lallier, R. 1985. Immunity to *Aeromonas salmonicida* in coho salmon (*Onchorynchus kisutch*) induced by modified Freund's complete adjuvant: Its non-specific nature and the probable role of macrophages in the phenomenon. Developmental & Comparative Immunology, 9:419-432.

211. Opperdoes, F.R., Borst, P., and Fonck, K. 1976. The potential use of inhibitors of glycerol-3-phosphate oxidase for chemotherapy of African trypanosomiasis. FEBS Letters, 62:169-172.

212. Ouaissi, A., Cornette, J., Schoneck, R., Plumas-Marty, B., Taibi, A., Loyens, M., and Capron, A. 1992. Fibronectin cleavage fragments provide a growth factor-like activity for the differentiation of *Trypanosoma cruzi* trypomastigotes to amastigotes. European Journal of Cell Biology, 59:68-79.

213. Ouaissi, M.A., Taibi, A., Cornette, J., Velge, P., Marty, B., Loyens, M., Esteva, M., Rizvi, F.S., and Capron, A. 1990. Characterization of major surface and excretory-secretory immunogens of *Trypanosoma cruzi* trypomastigotes and identification of potential protective antigen. Parasitology, 100:115-124.

214. Ouaissi, M.A., *et al.* 1991. *Trypanosoma cruzi* : A carbohydrate epitope defined by a monoclonal antibody as a possible marker of the acute phase of human Chaga's disease. American Journal of Tropical Medicine & Hygiene, 45:214-225.

215. Overath, P., Haag, J., Mameza, M.G., and Lischke, A. 1999. Freshwater fish trypanosomes: Definition of two types, host control by antibodies and lack of antigenic variation. Parasitology, 119:591-601.

216. Overath, P., Ruoff, J., Steirhof, Y.D., Haag, J., Tichy, H., Dykova, I., and Lom, J. 1998. Cultivation of bloodstream forms of *Trypanosoma carassii*, a common parasite of freshwater fish. Parasitology Research, 84:343-347.

217. Palenzuela, O., Sitja-Bobadilla, A., and Alvarez-Pellitero, P. 1997. *Ceratomyxa sparusaurati* (Protozoa: myxosporea) infections in cultured gilthead sea bream *Sparus aurata* (Pisces: teleostei) from Spain: Aspects of the host-parasite relationship. Parasitology Research, 83:539-548.

218. Pance, A. and Henriquez, D.A. 1992. Changes in proteolytic activity during the growth of *Trypanosoma cruzi* epimastigotes. Biochemistry International, 27:613-623.

219. Parsons, J.L., *et al.* 1988. The synthesis of XTT: A new tetrazolium reagent that is bioreducible to a water-soluble formazan. Journal of Heterocyclic Chemistry, 25:911-914.

220. Parsons, M. and Nielsen, B. 1990. Active transport of 2-deoxy-D-glucose in *Trypanosoma brucei* procyclic forms. Molecular & Biochemical Parasitology, 42:197-204.

221. Paterson, W.D. and Fryer, J.L. 1974. Immune response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* cells administered intraperitoneally in Freund's complete adjuvant. Journal Fisheries Research Board of Canada, 31:1751-1755.

222. Perlewitz, V.J. and Koch, A. 1985. Versuche zur optimierung der kulturbeingungen von human pathogenen Trypanosomen und Leishmanien. Angewandte Parasitologie, 26:185-191.

223. Pomport-Castillon, C., Gasc, C., and Romestand, B. 1999. IgM levels and specific antibody activity of the flounder (*Platichthys flesus* L.) following injection of microsporidian spores. Fish & Shellfish Immunology, 9:153-156.

224. Pulsford, A. and Matthews, R.A. 1991. Macrophages and giant cells associated with a microsporidian parasite causing liquefaction of the skeletal muscle of the Norway pout, *Trisopterus esmarkii* (Nilsson). Journal of Fish Diseases, 14:67-78.

225. Qadri, S.S. 1962. An experimental study of the life cycle of *Trypanosoma danilewskyi* in the leech, *Hemiclepsis marginata*. Journal of Protozoology, 9:254-258.

226. Rahmah, N. and Khairul Anuar, A. 1992. Comparisons of three forms of antigens in the demonstration of cell-mediated immune response in murine toxoplasmosis. Biochemical & Biophysical Research Communication, 189:640-644.

227. Raut, S.K. and Saha, T.C. 1998. Biological control of disease transmitting freshwater leeches *Hemiclepsis marginata marginata* (Muller) Annelida: Glossiphoniidae. Journal of the Bombay Natural History Society, 95:363-364.

228. Rijkers, G.T., Frederix-Wolters, E.M.H., and van Muiswinkel, W.B. 1980. The immune system of cyprinid fish, kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). Immunology, 41:91-97.

229. Rijkers, G.T., Wiegerinck, J.A.M., van Oosterom, R., and van Muiswinkel, W.B. 1980. Temperature dependence of humoral immunity in carp (*Cyprinus carpio*). in 1st Congress of Developmental and Comparative Immunology. Aberdeen: Pergamon Press.

230. Robertson, M. 1911. Transmission of flagellates living in the blood of certain freshwater fishes. Philosphical Transactions of the Royal Society, 202:29-50.

231. Roehm, N.W., Rodgers, G.H., Hatfield, S.M., and Glasebrook, A.L. 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. Journal of Immunological Methods, 142:257-265.

232. Roger, M. and Viens, P. 1987. *Trypanosoma musculi*:Influence of uric acid on *in vitro* formation of metacyclic trypomastigotes. Parasitology, 95:531-535.

233. Rouskova, H. and Kulda, J. 1983. Growth and transformation of *Trypanosoma danilewskyi* in a liquid medium. Folia Parasitologica, 30:295-301.

234. Safina, C. 1995. The world's imperiled fish. Scientific American, :46-53.

235. Sakai, D.K. 1984. Induction of nonspecific immunosuppression in a primitive teleost, rainbow trout (*Salmo gairdneri*). Developmental & Comparative Immunology, 8:569-578.

236. Sakai, D.K., 1992. Repertoire of complement in immunological defense mechanisms of fish. In Annual Review of Fish Diseases, eds Faisal, M. and Hetrick, F.M. Pergamon Press, New York, 1992, p. 223-247.

237. Salata, R.A., Martinez-Palomo, A., Cannales, L., Murray, H.W., Trevino, N., and Ravdin, J.I. 1990. Suppression of T-lymphocyte response to *Entamoeba histolytica* antigen by immune sera. Infection & Immunity, 58:3941-3946.

238. Salzman, T.A., Stella, A.M., Wider de Xifra, E.A., and Batlle, A.M.d.C. 1982. Porphyrin biosynthesis in parasitic hemoflagellates: Functional and defective enzymes in *Trypanosoma cruzi*. Comparative Biochemistry & Physiology, 72B:663-667.

239. Schaperclaus, W. 1992. Fish Diseases. 5th ed. Vol. 2. A.A. Balkema, Rotterdam.

240. Schell, D., Borowy, N.K., and Overath, P. 1991. Transferrin is a growth factor for the bloodstream form of *Trypanosoma brucei*. Parasitology Research, 77:558-560.

241. Schmidt, G.D. and Roberts, L.S. 1989. Foundations of Parasitology. 4th. Times Mirror/Mosby College Publishing, St. Louis.

242. Scott, P.A. and Sher, A., 1993. Immunoparasitology. In Fundamental Immunology, eds Paul, W.E. Raven Press Ltd., New York, 1993, p. 1179-1210.

243. Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D., and Boyd, M.R. 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in cuture using human and other cell lines. Cancer Research, 48:4827-4833.

244. Secombes, C.J. and Fletcher, T.C., 1992. The role of phagocytes in the protective mechanisms of fish. In Annual Review of Fish Diseases, eds Faisal, M. and Hetrick, F.M. Pergamon Press, New York, 1992, p. 53-71.

245. Serero, M. and Avtalion, R.R. 1978. Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates III. Establishment of immunological suppression in fish. Developmental & Comparative Immunology, 2:87-94.

246. Shapiro, S.Z. 1994. Failure of immunization with trypanosome endocytotic vesicle membrane proteins to provide nonvariant immunoprotection against *Trypanosoma brucei*. Parasitology Research, 80:240-244.

247. Sin, Y.M., Ling, K.H., and Lam, T.J. 1994. Passive transfer of protective immunity against ichthyophthiriasis from vaccinated mother to fry in tilapias, *Oreochromis aureus*. Aquaculture, 120:229-237.

248. Sin, Y.M., Ling, K.H., and Lam, T.J. 1996. Cell-mediated immune responses of goldfish, *Carassius auratus* (L.), to *Ichthyophthirius multifiliis*. Journal of Fish Diseases, 19:1-7.

249. Sitja-Bobadilla, A. and Alvarez-Pellitero, P. 1993. Pathologic effects of *Sphaerospora dicentrarchi* Sitja-Bobadilla and Alvarez-Pellitero, 1992 and *S. testicularis* Sitja-Bobadilla and Alvarez-Pellitero, 1990 (Myxosporea: Bivalvulida) parasitic in the Mediterranean sea bass *Dicentrarchus labrax* L. (Teleostei: Serranidae) and the cell-mediated immune reaction: A light and electron microscopy study. Parasitology Research, 79:119-129.

250. Sitja-Bobadilla, A. and Woo, P.T.K. 1994. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against the pathogenic haemoflagellate, *Cryptobia salmositica* Katz, and protection against cryptobiosis in juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum), inoculated with a live vaccine. Journal of Fish Diseases, 17:399-408.

251. Smolikova, V., Suchankova, E., and Lom, J. 1977. Growth of carp trypanosome, *T. danilewskyi* in fish tissue culture. Journal of Protozoology, 24:54A.

252. Southworth, G.C. and Read, C.P. 1969. Carbohydrate transport in *Trypanosoma gambiense*. Journal of Protozoology, 16:720-723.

253. Southworth, G.C. and Read, C.P. 1970. Specificity of sugar transport in *Trypanosoma gambiense*. Journal of Protozoology, 17:396-399.

254. Staines, G.J. and Woo, P.T.K. 1997. Immunization of susceptible chinook salmon (*Oncorhynchus tshawytscha*) against cryptobiosis. Bulletin of the Canadian Society of Zoologists, 28:123-124.

255. Steinhagen, D. 1997. Temperature modulation of the response of Ig-positive cells to *Goussia carpelli* (Protozoa: Apicomplexa) infections in carp, *Cyprinus carpio* L. Journal of Parasitology, 83:434-439.

256. Steinhagen, D. and Rombout, J.H.W.M. 1994. Response if Ig-positive cells to *Goussia carpelli* (Protozoa:Apicomplexa) infections in carp (*Cyprinus carpio* L.). Folia Parasitologica, 41:173-176.

257. Sternberg, J.M. and McGuigan, F. 1994. *Trypanosoma brucei*: Mammalian epidermal growth factor promotes the growth of the African trypanosome bloodstream form. Experimental Parasitology, 78:422-424.

258. Sullivan, J.J. 1982. Metacyclogenesis of *Trypanosoma cruzi in vitro*: A simplified procedure. Transactions of the Royal Society of Tropical Medicine, 76:300-303.

259. Sypek, J.P. and Burreson, E.M. 1983. Influence of temperature on the immune response of juvenile summer flounder, *Paralichthys dentatus*, and its role in the elimination of *Trypanoplasma bullocki* infections. Developmental & Comparative Immunology, 7:277-286.

260. Taibi, A., Espinoza, A.G., and Ouaissi, A. 1995. *Trypanosoma cruzi* : Analysis of cellular and humoral response against a protective recombinant antigen during experimental Chagas' disease. Immunology Letters, 48:193-200.

261. Taibi, A., *et al.* 1993. *Trypanosoma cruzi*: Immunity-induced in mice and rats by trypomastigote excretory-secretory antigens and identification of a peptide sequence containing a T cell epitope with protective activity. Journal of Immunology, 151:2676-2689.

262. Tait, A. 1980. Evidence for diploidy and mating in trypanosomes. Nature, 287:536-538.

263. Tait, A. and Turner, C.M.R. 1990. Genetic exchange in *Trypanosoma brucei*. Parasitology Today, 6:70-75.

264. Takle, G.B. and Young, A. 1988. A simple semi-defined culture medium for the growth of *Trypanosoma rangeli*. Transactions of the Royal Society of Tropical Medicine & Hygiene, 82:247.

265. Tandon, R.S. and Joshi, B.D. 1973. Studies on the physiopathology of blood of fresh water fishes infected with two new forms of trypanosomes. Zeitschrift fur Wissenschaftliche Zoologie, 185:207-221.

266. Tanner, M. 1980. Studies on the mechanisms supporting the continuous growth of *Trypanosoma* (*Trypanozoon*) *brucei* as bloodstream-like form *in vitro*. Acta Tropica, 37:203-220.

267. ter Kuile, B.H., 1991. Glucose uptake mechanisms as potential targets for drugs against trypanosomatids. In Biochemical Protozoology, eds Coombs, G. and North, M. Taylor & Francis, London, 1991, p. 359-366.

268. ter Kuile, B.H. 1997. Adaptation of metabolic enzyme acticities of *Trypanosoma brucei* promastigotes to growth rate and carbon regimen. Journal of Bacteriology, 179:4699-4705.

269. ter Kuile, B.H. and Opperdoes, F.R. 1991. Glucose uptake by *Trypanosoma brucei* Rate-limiting steps in glycolysis and regulation of the glycolytic flux. Journal of Biological Chemistry, 266:857-862.

270. ter Kuile, B.H. and Opperdoes, F.R. 1992. Comparative physiology of two protozoan parasites, *Leishmania donovani* and *Trypanosoma brucei*, grown in chemostats. Journal of Bacteriology, 174:2929-2934.

271. Thomas, P.T. and Woo, P.T.K. 1990. *In vivo* and *in vitro* cell-mediated immune responses of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Cryptobia salmositica* Katz, 1951 (Sarcomastigophora: Kinetoplastida). Journal of Fish Diseases, 13:423-433.

272. Thomaz, N. and Deane, M.P. 1990. Continous growth and differentiation of *Trypanosoma* (*Megatrypanum*) *freitasi* Rego, Magalhaes & Siqueria, 1957, *in vitro*. Journal of Protozoology, 37:40-43.

273. Thomson, J.D. 1908. Cultivation of the trypanosome found in the blood of the gold-fish. Journal of Hygiene, 8:75-82.

274. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences USA, 76:4350-4354.

275. van Duijn, C. 1967. Diseases of Fishes. 2nd ed. Iliffe Books Ltd., London.

276. Vethamany-Globus, S., Globus, M., and Fraser, I. 1977. Effects of tricaine methane sulfonate (M.S.222) on the blood glucose levels in adult salamanders (*Diemictylus viridescens*). Experientia, 33:1027.

277. Viens, P., Lajeunesse, M.C., Richards, R., and Targett, G.A.T. 1977. *Trypanosoma musculi*: *In vitro* cultivation of blood forms in cell culture media. International Journal for Parasitology, 7:109-111.

278. Vincendeau, P., Guillemain, B., Daulouede, S., and Ripert, C. 1986. *In vitro* growth of *Trypanosoma musculi*: Requirements of cells and serum free culture medium. International Journal for Parasitology, 16:387-390.

279. Wahli, T. and Matthews, R.A. 1999. Ichthyophthiriasis in carp *Cyprinus carpio*: Infectivity of trophonts prematurely exiting both the immune and non-immune host. Diseases of Aquatic Organisms, 36:201-207.

280. Wahli, T. and Meier, W., 1985. Ichthyophthiriasis in trout: Investigation of natural defense mechanisms. In Fish & Shellfish Pathology, eds Ellis, A.E. Academic Press, London, 1985, p. 347-352.

281. Wahli, T., Meier, W., and Pfister, K. 1986. Ascorbic acid induced immune-mediated decrease in mortality in *Ichthyophthirius multifiliis* infected rainbow-trout (*Salmo gairdneri*). Acta Tropica, 43:287-289.

282. Wahli, T., Verlhac, V., Gabaudan, J., Schuep, W., and Meier, W. 1998. Influence of combined vitamins C and E on non-specific immunity and disease resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases, 21:127-137.

283. Wang, R.The influence of steroids on host defense against *Trypanosoma danilewskyi* (Laveran & Mesnil, 1904) in the goldfish *Carassius auratus* (L.) *Department of Biological Sciences*. 1994, University of Alberta: Edmonton.

284. Wang, R. and Belosevic, M. 1994. Cultivation of *Trypanosoma danilewskyi* (Laveran & Mesnil, 1904) in serum-free medium and assessment of the course of infection in goldfish, *Carassius auratus* (L.). Journal of Fish Diseases, 17:47-56.

285. Wang, R., Neumann, N.F., Shen, Q., and Belosevic, M. 1995. Establishment and characterization of a macrophage cell line from the goldfish. Fish & Shellfish Immunology, 5:329-346.

286. Wang, W. and Chadee, K. 1992. *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages *in vitro* and *in vivo*. Immunology, 76:242-250.

287. Wang, W. and Chadee, K. 1995. *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatability complex Ia molecule and I-A beta mRNA expression by a prostaglandin E2-dependent mechanism. Infection & Immunity, 63:1089-1094.

288. Webster, P. and Russell, D.G. 1993. The flagellar pocket of trypanosomatids. Parasitology Today, 9:201-206.

289. Wehnert, S. and Woo, P.T.K. 1981. The immune responses of *Salmo gairdneri* during *Trypanoplasma salmositica* infection. Bulletin of the Canadian Society of Zoologists, 12:100.

290. Wehnert, S.D. and Woo, P.T.K. 1980. *In vivo* and *in vitro* studies on the host specificity of *Trypanoplasma salmositica*. Journal of Wildlife Diseases, 16:183-187.

291. Wiegertjes, G.F., A., G., and van Muiswinkel, W.B. 1995. Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.). Veterinary Immunology & Immunopathology, 47:153-161.

292. Wiegertjes, G.F., Bongers, A.B.J., Voorthuis, P., Zandieh Doulabi, B., Groeneveld, A., van Muiswinkel, W.B., and Stet, R.J.M. 1996. Characterization of isogenic carp (*Cyprinus carpio* L.) lines with genetically determined high or low antibody production. Animal Genetics, 27:313-319.

293. Wilson, M.R., Wang, A., Fish, W.W., and Warr, G.W. 1985. Anomalous behavior of goldfish IgM heavy chain in sodium dodecylsulfate polyacrlamide gel electrophoresis. Comparative Biochemical Physiology, 82B:41-49.

294. Wita, I. and Ovcharenko, M. 1997. Occurence of trypanosomes *Trypanosoma carassii* in the blood of roach *Rutilus rutilis* and tench *Tinca tinca*. Wiadomosci-Parazytologiczna, 43:385-392.

295. Wolf, K. and Markiw, M.E. 1982. Ichthyphthiriasis: Immersion immunization of rainbow trout (*Salmo gairdneri*) using *Tetrahymena thermophila* as a protective immunogen. Canadian Journal of Fisheries & Aquatic Sciences, 39:1722-1725.

296. Woo, P.T.K. 1969. The haematocrit centrifuge for the detection of trypanosomes in blood. Canadian Journal of Zoology, 47:921-923.

297. Woo, P.T.K. 1979. *Trypanoplasma salmositica*: Experimental infections in rainbow trout, *Salmo gairdneri*. Experimental Parasitology, 47:36-48.

298. Woo, P.T.K. 1981. Acquired immunity against *Trypanosoma danilewskyi* in goldfish, *Carassius auratus*. Parasitology, 83:343-346.

299. Woo, P.T.K. 1981. *Trypanosoma danilewski*: A new multiplication process for *Trypanosoma* (Protozoa: Kinetoplastida). Journal of Parasitology, 67:522-526.

300. Woo, P.T.K. 1990. MISET: An immunological technique for the serodiagnosis of *Cryptobia salmositica* (Sarcomastigophora: Kinetoplastida) infection in *Oncorhynchus mykiss*. Journal of Parasitology, 76:389-393.

301. Woo, P.T.K., 1995. ed. Fish Diseases and Disorders. Protozoan and metazoan infections, Vol. 1. CAB International: Wallingford. 808.

302. Woo, P.T.K. 1999. The use of monoclonal antibody probes in salmonid cryptobiosis. Aquaculture, 177:311-324.

303. Woo, P.T.K. and Black, G.A. 1984. *Trypanosoma danilewskyi*: Host specificity and host's effect on morphometrics. Journal of Parasitology, 70:788-793.

304. Woo, P.T.K. and Jones, R.M., 1989. The piscine immune system and the effects of parasitic protozoans on the immune response. In Current Concepts in Parasitology, eds Ko, R.C. Hong Kong University Press, Hong Kong, 1989, p. 267.

305. Woo, P.T.K., Leatherland, J.F., and Lee, M.S. 1987. *Cryptobia salmositica*: Cortisol increases the susceptibility of *Salmo gairdneri* Richardson to experimental cryptobiosis. Journal of Fish Diseases, 10:75-83.

306. Woo, P.T.K. and Li, S. 1990. *In vitro* attenuation of *Cryptobia salmositica* and its use as a live vaccine against cryptobiosis in *Oncorhynchus mykiss*. Journal of Parasitology, 76:752-755.

307. Woo, P.T.K. and Poynton, S.L., 1995. Diplomonadidia, Kinetoplastida, and Amoebida (Phylum Sarcopmastigophora). In Fish Disease and Disorders, eds Woo, P.T.K. CAB International, Wallingford, 1995, p. 27-96.

308. Woo, P.T.K. and Rogers, D.J. 1974. A statistical study of the sensitivity of the haematocrit centrifuge technique in the detection of trypanosomes in blood. Transactions of the Royal Society of Tropical Medicine & Hygiene, 68:319-326.

309. Woo, P.T.K., Wehnert, S.D., and Rodgers, D. 1983. The susceptibility of fishes to haemoflagellates at different ambient temperatures. Parasitology, 87:385-392.

310. Yoshikawa, H., Furuki, J., Takahashi, Y., Morioka, H., and Yoshida, Y. 1990. Freeze-fracture study of the bloodstream form of *Trypanosoma brucei gambiense*. Journal of Protozoology, 37:27-32.

APPENDIX A

ELSEVIER
SCIENCE

Elsevier Science

Global Rights
The Boulevard
Langford Lane
Kidlington
Oxford OX5 1GB
England

Tel (+44) (0) 1865 843830
Fax (+44) (0) 1865 853333
E-mail: permissions@elsevier.co.uk
Home Page: www.elsevier.com

FCR/sjg/aug00/539
18 August 2000

Dr. Diane R Bienek
University of Alberta
CW-405 Biological Sciences Centre
Edmonton, Alberta T6G 2E9
Canada

Dear Dr. Bienek

DEVELOPMENTS IN AQUACULTURE & FISHERIES SCIENCE, Vol 23, 1992, Lom, "Protozoan parasites of..."

As per your letter dated 02 August 2000, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made as follows:

   "Reprinted from Journal title, Volume number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier Science".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for the National Library of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Frances Rothwell (Mrs)
Global Rights Manager

The processing of permission requests for all Elsevier Science (including Pergamon imprint) journals has been centralised in Oxford, UK. Your future requests will be handled more quickly if you write directly to: Subsidiary Rights Department, Elsevier Science, PO Box 800, Oxford OX5 1DX, UK.
Fax: 44-1865 853333; e-mail: permissions@elsevier.co.uk

Imprints:
Elsevier
Pergamon
North-Holland
Excerpta Medica

APPENDIX B

TABLE OF CONTENTS

Appendix B. Analysis of active immunization of goldfish for protection against *T. danilewskyi* 191
Experimental design 191
Results 191
  Lysate of parasites 191
    Intravenous administration 191
    Intramuscular administration 192
    Intraperitoneal administration (no adjuvant) 193
    Intraperitoneal administration (Freund's incomplete adjuvant) 194
    Intraperitoneal administration (Freund's complete adjuvant) 194
  Water-soluble extracts 195
    Intravenous administration 195
    Intraperitoneal administration (no adjuvant) 197
    Intraperitoneal administration (Freund's incomplete adjuvant) 197
    Intraperitoneal administration (Freund's complete adjuvant) 198
  Detergent-soluble extracts 199
    Intravenous administration 199
    Intramuscular administration 200
    Intraperitoneal administration (no adjuvant) 200
    Intraperitoneal administration (Freund's incomplete adjuvant) 201
    Intraperitoneal administration (Freund's complete adjuvant) 201
    Gel electrophoresis/immunoblotting 202

APPENDIX B

ANALYSIS OF ACTIVE IMMUNIZATION OF GOLDFISH FOR PROTECTION AGAINST *TRYPANOSOMA DANILEWSKYI*

EXPERIMENTAL DESIGN

Please refer to Chapter V for the experimental design relevant to this appendix.

RESULTS

Lysate of parasites

Intravenous administration. Preparations of trypanosome lysate, administered intravenously, did not protect fish against *T. danilewskyi*. All fish inoculated intravenously with parasite lysate or control solution survived the challenge infection (Fig. B-1A). Seven days postinfection, the prevalence of infection was 100% in both of these groups (Fig. B-1B). Analysis of data (14, 28, or 56 dpi) demonstrated that the number of parasites ml^{-1} blood were significantly greater ($P < 0.04$) than in control fish (Fig. B-1C). By 56 dpi, all immunized fish were still infected with a mean number of 6 X 10^5 parasites ml^{-1} blood. In contrast, two of five control fish eliminated trypanosomes from the blood. The remaining control fish had an intensity of 3.72 X 10^3 parasites ml^{-1}. Only a weak positive correlation was drawn between parasitemias and the antibody response of fish inoculated intravenously with trypanosome lysate or control medium (r = 0.44 and 0.32, respectively) (Table B-2).

Although the ELISA values of the immunized group was two-fold greater than that observed in the control group (0 dpi), intravenous immunization with trypanosome lysate did not significantly affect the humoral immune response (Fig. B-1E). After exposure to *T. danilewskyi*, the mean ELISA values of immunized fish were consistently higher than those of the control group. The greatest difference (three-fold) between these groups was observed on 7 dpi ($P < 0.05$). By 14 dpi, the mean ELISA values increased in both the experimental and control group. However, the percent of control value (i.e., 80%) suggests that the increase observed in the control fish was greater than that of the immunized group (Table B-1). For the rest of the experiment, the percent of control values remained at 60%, indicating that the immunized challenged fish had a lower antibody response than that of the control fish given a primary infection.

For all inoculum routes, the mean red cell volume of fish immunized with trypanosome lysate was like that observed in the respective control group (Fig. B-1D to B-5D). When comparing mean red cell volumes over time, a few differences were observed between the administration routes. Notwithstanding, these data are presented in this section. No statistical difference in the mean red cell volume was observed during the first week of infection (Fig, B-1D, B-2D, B-4D, and B-5D), except for the decline ($P < 0.01$) observed in the group given an intraperitoneal inoculation (Fig. B-3D). Groups given intramuscular or intraperitoneal (with FIA) inoculations had similar red cell volumes on 14 dpi as that recorded on 28 and 56 dpi (Fig. B-2D and B-4D). In contrast, the mean red cell volumes of the other routes of administration increased significantly ($P < 0.002$) over time (14 vs 56 dpi). Differences ($P < 0.04$) between the non-immunized infected group and the non-immunized uninfected group were observed early in the infection (Fig. 5-8D). For these fish, the lowest red cell volume of 30% was observed on 14 dpi. Afterwards, the mean value increased ($P < 0.01$) steadily with time.

Intramuscular administration. Intramuscular immunization with trypanosome lysate did not confer protection against subsequent challenge. Death only occurred in one control fish that suffered massive parasitemia (Fig. B-2A). Although there was no significant differences in the mean parasitemia of immunized or control fish; administration of control solution facilitated a marked reduction (75%) in the prevalence of infection by day 56 (Fig. B-2B and B-2C). During the infection, the change in the ELISA values observed in the immunized group did not correlate significantly with the number of parasite ml-1 blood (Table B-2). Conversely, heavier trypanosome infections in the control group correlated ($P < 0.001$) with an increase in antibodies.

Prior to *T. danilewskyi* infection, the humoral response of fish immunized intramuscularly with trypanosome lysate was similar to that of fish inoculated with control solution (Fig. B-2E). During the course of infection, the mean ELISA values of the control group was noticeably greater than in the immunized group. However, these differences were not significant, as the responses between cohorts were exceedingly heterogeneous. For instance, relative to that observed on 0 dpi, the ELISA values of individual control fish on 28 dpi ranged between 60 and 290% (median = 125%). As illustrated by the mean ELISA values, the percent of control value confirmed that detection of parasite specific antibodies in immunized fish was 30 to 60% lower than that detected in control fish (Table B-1).

Intraperitoneal administration (no adjuvant). Similar to that observed for other routes of administration, intraperitoneal inoculation of trypanosome lysate had little effect on the outcome of the disease. Although all fish had become infected with *T. danilewskyi*, no mortality occurred in the immunized or control group (Fig. B-3A and B-3B). The course of infection was very similar in both groups (Fig. B-3C). In brief, peak parasitemia was observed 14 dpi, thereafter a marked reduction in the mean number of parasites was observed by 28 and 56 dpi. For all groups given intraperitoneal injections (i.e., with or without adjuvant) of parasite lysate or control solution, no significant correlation was drawn between the abundance of parasites and the antibody response (Table B-2).

The mean ELISA value (0 dpi) was not significantly affected by intraperitoneal immunization with trypanosome lysate (Fig. B-3E). Further, the antibody response of immunized and control fish were similar throughout the infection. Although the mean ELISA value of these two groups was not significantly different, the percent of control value suggests that the responsiveness of immunized fish was consistently lower than that of the control group (Table B-1).

Intraperitoneal administration (FIA). Although insignificant, trypanosome lysate given with FIA elicited a small degree of protection. The prevalence of infection of the immunized group was at least 20% lower than in control fish on 7, 14, and 28 dpi (Fig. B-4B). The increase in the prevalence in immunized fish between 28 and 56 dpi may be attributed to the detection limit of the haematocrit centrifuge technique. Though the mean number of trypanosomes ml^{-1} blood between treatment groups was not statistically different, the response of individual fish was noteworthy (Fig. B-4C). Three of five immunized fish had low parasitemia that never exceeded 2.5×10^5 *T. danilewskyi* ml^{-1} blood. In contrast, four of the five control fish had a mean parasite intensity of 2.71×10^7 and 1.63×10^6 ml^{-1} blood on 7 and 14 dpi, respectively (data not shown). All fish in this trial survived for the duration of the experiment (Fig. B-4A).

Much like that reported in the intraperitoneal experiment (no adjuvant), immunization with trypanosome lysate suspended in FIA of *T. danilewskyi* challenge did not significantly affect the antibody response of fish (Fig. B-4E). During the first week of the infection, both groups showed a decline in the presence of antibodies. Thereafter, the mean ELISA values increased and remained at values comparable to that observed on 0 dpi.

Intraperitoneal administration (FCA). The most notable difference between control fish and those immunized with a suspension of trypanosome lysate and FCA was observed in the prevalence of infection (Fig. B-5B). At 7 dpi, *T. danilewskyi* was detected in the blood of all fish. Some fish in the immunized group cleared the infection from the blood by 14 and 28 dpi, whereas all of the control fish remained infected. On the last day of the experiment, no parasites were detected in the blood of 60% and 40% of the immunized and control fish, respectively. In spite of these differences, the parasitemias of the two groups were comparable (Fig. B-5C). Moreover, no mortality was observed in either treatment group (Fig. B-5A). Regarding the antibody response, statistical analyses of data (collected from fish immunized with trypanosome lysate suspended in FCA) was like that reported for the other groups given intraperitoneal inoculations, with one exception (Fig. B-5E). Unlike that observed for the other routes of administration, the percent of control value ranged between 170 and 300% (Table B-1).

Water-soluble extracts

Intravenous administration. Intravenous immunization of trypanosome water-soluble molecules appeared to affect the goldfish adversely. One control fish died during the immunization period (13 days after inoculation). Otherwise, no mortality was observed in the control group (Fig. B-6A). By 56 dpi, two heavily infected immunized fish had died. In the immunized and control group, the prevalence of infection reached a maximum of 80% on 7 dpi. (Fig. B-6B). There was a single fish in each group that did not develop a patent infection. On the last day of observation, one of the four surviving immunized fish remained infected (5×10^7 parasite ml^{-1} blood). Conversely, all fish in the control group had cleared the infection. Although not significantly different, the mean parasitemia of immunized fish were consistently higher than that observed in the control group (Fig. B-6C). The abundance of parasites given intravenous injections did not correlate significantly with the change of parasite-specific antibodies (Table B-4).

Analysis of mean ELISA values suggested that intravenous administration of water-soluble molecules suppressed the humoral immune response (Fig. B-6E). The mean response of antiserum, collected prior to *T. danilewskyi* exposure, was significantly lower ($P < 0.05$) than noticed in fish given control solution.

The mean ELISA value of the control group was consistently two-fold higher than that of the immunized group; however, these analyses revealed no significant difference (Fig. B-6E). Relative to that observed 0 dpi, the mean ELISA value of both groups increased two-fold during the course of infection. However, the percent of control values suggested that the humoral immune response of immunized fish was initially suppressed

(Table B-3). Also, after the peak antibody response at 14 dpi, a marked decline in the percent of control value was noted.

From the course of infection and the antibody response of immunized fish, it was difficult to conclude if any fish received an unsuccessful inoculation. For instance, the parasite abundance of intravenously immunized fish fell into two general groups. Three of the six experimental fish had a course of infection that was comparable to that of control fish. The remaining experimental fish had considerably higher parasitemia that persisted throughout the experiment. Although this variability was commonly observed in goldfish given a primary infection (see Chapter III), these observations may suggest that intravenous administration was successful in 50% of the fish and that this immunization increased the susceptibility of the fish to *T. danilewskyi*. ELISA values of individual fish (relative to that observed 0 dpi) did not support this theory, as fluctuations of the majority of samples ranged between 50 and 230%. Only one of the three fish, presumed to have received an unsuccessful inoculation, had ELISA values exceeding 300%.

Regardless of the immunization route, the red cell volume of fish (given water-soluble molecules or control medium) followed a similar pattern. Consequently, these data are presented in this section. No significant differences were observed when the red cell volume of experimental and control fish were compared (Fig. B-6D to B-9D). Likewise, no significant differences were observed when the mean red cell volume of the non-immunized infected group was compared to that of the non-immunized uninfected group (Fig. 5-9D). Examination of the time course indicated that the mean red cell volume of all groups decreased significantly ($P < 0.008$) during the first week of the trypanosome infection. Except for the significant increase ($P < 0.009$) observed in the red cell volume of fish given an intravenous inoculation (Fig. B-6D), the mean values observed on 14 dpi were comparable to that observed on 28 dpi (Fig. B-7D, B-8D, and B-9D). By 56 dpi, the mean red cell volume of the fish had increased ($P < 0.02$) and attained values comparable to that observed prior to *T. danilewskyi* exposure.

Intraperitoneal administration (no adjuvant). Water-soluble molecules administered intraperitoneally did not confer protection against the challenge inoculum. During the course of infection, mortality was only observed in the control group on 14 dpi (Fig. B-7A). Although all fish developed a patent infection, the prevalence of infection reached only 80% (Fig. B-7B). Curiously, four of the five surviving control fish cleared the infection by 14 dpi. In comparison, five of the six immunized fish retained the infection. At the onset of the infection, the mean number of parasites of the two groups was very similar (Fig. B-7C). Later in the infection (14 to 56 dpi) fish immunized intraperitoneally with water-soluble molecules had ≥ 60% ($P < 0.04$) more parasites than those inoculated with control medium. Nevertheless, the parasitemia of fish given intraperitoneal inoculations (no adjuvant) did not correlate well with the change of ELISA values over time (Table B-4).

Intraperitoneal immunization alone (0 dpi) resulted in higher ($P < 0.05$) antibody responses than that observed in control fish (Fig. B-7E). More specifically, the increase of the mean ELISA value of immunized fish was greater than two-fold. Following *T. danilewskyi* challenge, the mean ELISA values were consistently greater than that observed in control fish, although these comparisons were not significant. Relative to the control group (percent of control value), the humoral response of the immunized fish was suppressed by ~30% at the onset of the infection (Table B-3). Thereafter, the ELISA values increased moderately and followed a bimodal distribution. Namely, peak antibody responses of similar magnitude were observed 14 and 56 dpi.

Intraperitoneal administration (FIA). Infection dynamics of fish immunized with water-soluble molecules suspended in FIA was like that observed in the control group. Although all fish developed a patent infection, only one heavily infected control fish died (Fig. B-8A). The prevalence of infection in both groups was similar, except that the immunized group was lower by 20% on 7, 14, 28, and 56 dpi (Fig. B-8B). Similar to that observed in the other treatment groups, water-soluble extracts given with FIA did not significantly affect parasite numbers within the blood (Fig. B-8C). As reported with the treatment group receiving whole dead parasites, a significant correlation ($P < 0.05$) was detected between the abundance of parasites and the antibody response of immunized fish (Table B-4). The resulting correlation coefficient of the group receiving FIA with control medium was not significant.

Although the mean ELISA value of experimental fish was noticeably increased, this response was not significantly affected by immunization or *T. danilewskyi* infection (Fig. B-8E). As observed with intraperitoneal immunization (without adjuvant) little variability was observed between control fish given a primary infection. In contrast, the response of immunized fish was remarkably heterogeneous. When adjusted for the mean ELISA value of control fish, the antibody response of experimental fish rose moderately (approximately 140% of control) and peaked at 14 dpi (Table B-3). By 28 and 56 dpi, the percent of control value declined to approximately 80% and 60%, respectively.

Intraperitoneal administration (FCA). Immunization of fish with water-soluble molecules suspended in FCA produced an unfavorable outcome. While all control fish survived the trypanosome infection, 40% mortality occurred in the immunized group (Fig. B-9A). With the exception of one immunized fish, *T. danilewskyi* was detected in the blood of all fish. Notwithstanding, the prevalence of infection in the immunized group was greater on 4, 14, and 28 dpi (Fig. B-9B). Although not significantly different from the control group, immunization resulted in heavier parasitemia (Fig. B-9C). As observed previously (i.e., sodium perchlorate-treated trypanosome experiment), a positive correlation ($P < 0.0002$) between the trypanosome number and the change in ELISA values was only observed in the group that received FCA mixed with control solution (Table B-4).

The mean ELISA values of immunized infected fish increased more rapidly at the onset of infection than that of the control group (Fig. B-9E). Correspondingly, the peak antibody response in the control fish occurred 2 wk after that in the experimental group. For each group, the highest ELISA value was approximately two-fold greater than that observed on 0 dpi. However, because of the variability, these comparisons were not significant. The percent of control values rose 20% during the first 14 days of the infection. Thereafter, the response was dramatically suppressed (~ 60% of the control).

Detergent-soluble extracts

Intravenous administration. Detergent-soluble molecules administered intravenously did not significantly affect the infection dynamics in goldfish. Except for one heavily infected immunized fish, all fish survived (Fig. B-10A). Although the prevalence of infection in both groups reached 100% by 4 dpi, the immunized group began clearing the infection faster than the control fish (Fig. B-10B). Specifically, two lightly infected immunized fish (< 2500 parasite ml^{-1} blood) eliminated the trypanosomes from the peripheral blood by 7 and 28 dpi. In contrast, the control group started clearing the infection on 28 and 56 dpi. Though not significant, detergent-solubilized molecules administered intravenously conferred partial protection against *T. danilewskyi* challenge, as the number of parasites was approximately 75% of the control (Fig. B-10C). This reduction in immunized fish was primarily due to data collected from the two aforementioned fish. These observations may suggest that intravenous immunization was successful in 40% of the fish and that this immunization markedly increased the resistance of fish. However, it was more likely that these observations reflect the heterogeneity of the immune response, as other administration routes had a similar magnitude of variability (Fig, B-11C to B-14C).

The administration route of detergent-soluble molecules affected the red cell volumes differently. For this reason, these data will be presented separately in each subsection. The mean red cell volume of intravenously immunized fish was comparable to that in the control group (Fig. B-10D). By 7 dpi, these fish had a slight (3 to 4%) but significant ($P < 0.0002$) decrease in the red cell volume. Fourteen days after *T. danilewskyi* challenge, these values increased ($P < 0.02$) with time to values that were similar to that observed 0 dpi.

Intramuscular administration. An indication of protection was evident when the infection dynamics of immunized fish was compared to that of control fish; howbeit, no statistical differences were drawn. Between 7 and 28 dpi, all control fish were infected with *T. danilewskyi* (Fig. B-11B). In contrast, the prevalence of infection in the experimental group dropped from 100% to 50% by 28 dpi. This value fluctuated later in the infection, inasmuch as two of the fish had parasitemias that remained around the detection limit. As illustrated, the mean parasitemia of immunized fish was 25% lower than that in the control group (Fig. B-11C). During the course of infection (28 and 56 dpi), one fish in each treatment group died (Fig. B-11A).

As with the intravenous route, no significant differences in the red cell volume were drawn between experimental and control fish given an intramuscular inoculation (Fig. B-11D). Also as previously observed, the mean value in intramuscularly inoculated fish decreased slightly ($P < 0.02$) during the first week of the infection. A significant increase ($P < 0.04$) in the mean red cell volume was recorded by 56 dpi.

Intraperitoneal administration (no adjuvant). Similar to that observed with intramuscular immunization, intraperitoneal administration of detergent-soluble molecules seemed to confer partial protection. By 56 dpi, death only occurred in one heavily infected immunized fish (Fig. B-12A). During the first 2 wk of the infection, the prevalence of infection in the control group was 100%. By 56 dpi, no trypanosomes were found in the blood of two of these five fish (Fig. B-12B). Although all immunized fish had developed a patent infection, the prevalence of infection reached a maximum of 80%. On the last day of observation, three of the four surviving immunized fish had cleared the infection. When comparing the abundance of parasites in immunized and control fish, no significant differences were found (Fig. B-12C). During the first 2 wk, the notable difference in the mean number of parasites was primarily due to a single fish that had developed a light infection (< 750 parasites ml^{-1} blood).

A significant difference ($P < 0.02$) between the mean red cell volume of intraperitoneally immunized and control fish was only observed with results collected after the first week of the infection (Fig. B-12D). The 3% decrease observed in these groups, during the first week, was significant ($P < 0.002$). Thereafter, there was a notable but insignificant increase in the mean red cell volume.

Intraperitoneal administration (FIA). Immunization with detergent-soluble extracts and FIA affected fish unfavorably, inasmuch as mortality and prevalence of infection was greater in these fish than in the control group (Fig. B-13A and B-13B). Immunized fish had a course of infection that was similar to that observed in a primary infection. However, fish in the corresponding control group had significantly ($P < 0.02$) fewer trypanosomes present in the blood.

Unlike that observed with the other administration protocols, the mean red cell volume in fish immunized intraperitoneally was consistently greater ($P < 0.03$) than that observed in the control group (Fig. B-13D). However, comparison of the data over time resulted in a trend similar to that previously reported. Briefly, the significant decrease ($P < 0.02$), observed in the first week, was followed by a restoration to that observed on 0 dpi.

Intraperitoneal administration (FCA). When comparing infection dynamics of experimental fish, inoculated with detergent-soluble molecules suspended in FCA, with the control group, no marked differences were observed. No mortality was observed in either group (Fig. B-14A). One fish in each group never developed a patent infection. Consequently, the prevalence of infection reached a maximum of 80% (Fig. B-14B). Although both groups had fish with parasitemias that hovered around the detection limit, a fluctuation (i.e., rebound) in the prevalence of infection was only observed in the control group. By 56 dpi, the prevalence of infection in the experimental and control group was 40% and 60%, respectively. The abundance of trypanosomes in the blood of immunized and control group was comparable (Fig. B-14C).

For the duration of the experiment, the mean red cell volumes of fish given intraperitoneal inoculations with detergent-soluble molecules and FCA were virtually equivalent to that observed in the control group (Fig. B-14D). Over the first week infection, a modest (3 to 5%) decrease ($P < 0.05$) in the mean value was recorded. A significant recovery ($P < 0.007$) in the red cell volume was detected between 14 and 56 dpi.

Gel electrophoresis/immunoblotting. Attempts to optimize the ELISA conditions of detergent-soluble molecules of *T. danilewskyi* were unsuccessful. In all trials, the optical density value of wells containing the antigen equivalence of 1 X 10^5, 1 X 10^6, or 2 X 10^6 parasites never exceeded that of the negative control wells. I suspected that the excess and molecule-bound detergent interfered with the binding of the parasite extract to the ELISA plate.

To determine the antigenic profile of the detergent solubilized integral membrane molecules, these extracts and solubilization buffer (control solution) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and stained with Ponceau or Coomassie stain. Staining of the membrane containing detergent-solubilized molecules disclosed a diffuse 50-53 kDa band. Conversely, no bands were observed on the control membranes. In a preliminary experiment, nitrocellulose membranes were tested with immune (56 dpi) or normal fish serum. Detergent-solubilized molecules of molecular weight 44 and 51 kDa were recognized by immune serum (data not shown). Conversely, these bands were not visible on the lanes reacted with normal fish serum. At similar antibody concentrations, no bands were observed on negative control lanes in which the fish serum, anti-carp IgM hybridoma supernatant, or the enzyme-labeled antibodies were omitted. In an attempt to reduce the nonspecific background staining, a different blocking procedure was tested. However, because of the limited amount of fish serum available, a satisfactory duplicate blot could not be obtained.

Table B-1. Percent of control value[a] of the antibody response of fish immunized with *Trypanosoma danilewskyi* lysate.

Administration route	TIME (days postinfection)			
	7	14	28	56
Intravenous	164	81	61	60
Intramuscular	66	47	43	68
Intraperitoneal	87	81	45	87
Intraperitoneal/FIA[b]	122	97	67	77
Intraperitoneal/FCA[c]	300	281	170	199
Not immunized	81	116	292	565

[a] ELISA values transformed into the percent increase or decrease of that observed 0 dpi, which equals 100%

[b] Freund's incomplete adjuvant

[c] Freund's complete adjuvant

Table B-2. Correlation coefficient (r) between parasitemia and the antibody response of fish immunized with *Trypanosoma danilewskyi* lysate.

Treatment	Administration Route	r	*P*-value
Parasite lysate	Intravenous	0.44	N.S.[a]
Control solution	Intravenous	0.32	N.S.
Parasite lysate	Intramuscular	0.14	N.S.
Control solution	Intramuscular	0.68	$P < 0.001$
Parasite lysate	Intraperitoneal	0.23	N.S.
Control solution	Intraperitoneal	0.18	N.S.
Parasite lysate/FIA[b]	Intraperitoneal	0.02	N.S.
Control solution/FIA[c]	Intraperitoneal	0.18	N.S.
Parasite lysate/FCA	Intraperitoneal	0.04	N.S.
Control solution/FCA	Intraperitoneal	0.47	N.S.
Non-immunized infected	N/A[d]	0.02	N.S.

[a] not significant
[b] Freund's incomplete adjuvant
[c] Freund's complete adjuvant
[d] not applicable

Table B-3. Percent of control value[a] of the antibody response of fish immunized with water-soluble molecules of *Trypanosoma danilewskyi*.

Administration route	TIME (days postinfection)				
	4	7	14	28	56
Intravenous	87	105	186	68	91
Intraperitoneal	72	67	134	82	138
Intraperitoneal/FIA[b]	78	113	145	84	62
Intraperitoneal/FCA[c]	106	104	120	36	42
Not immunized	115	98	197	104	46

[a] ELISA values transformed into the percent increase of decrease of that observed 0 dpi, which equals 100%

[b] Freund's incomplete adjuvant

[c] Freund's complete adjuvant

Table B-4. Correlation coefficient (r) between parasitemia and the antibody response of fish immunized with water-soluble molecules (WSM) of *Trypanosoma danilewskyi.*

Treatment	Administration Route	r	*P*-value
WSM	Intravenous	0.12	N.S.[a]
Control solution	Intravenous	0.14	N.S.
WSM	Intraperitoneal	0.09	N.S.
Control solution	Intraperitoneal	0.15	N.S.
WSM/FIA[b]	Intraperitoneal	0.37	$P < 0.05$
Control solution/FIA[c]	Intraperitoneal	0.33	N.S.
WSM/FCA	Intraperitoneal	0.01	N.S.
Control solution/FCA	Intraperitoneal	0.63	$P < 0.0002$
Non-immunized infected	N/A[d]	0.37	$P < 0.05$

[a] not significant
[b] Freund's incomplete adjuvant
[c] Freund's complete adjuvant
[d] not applicable

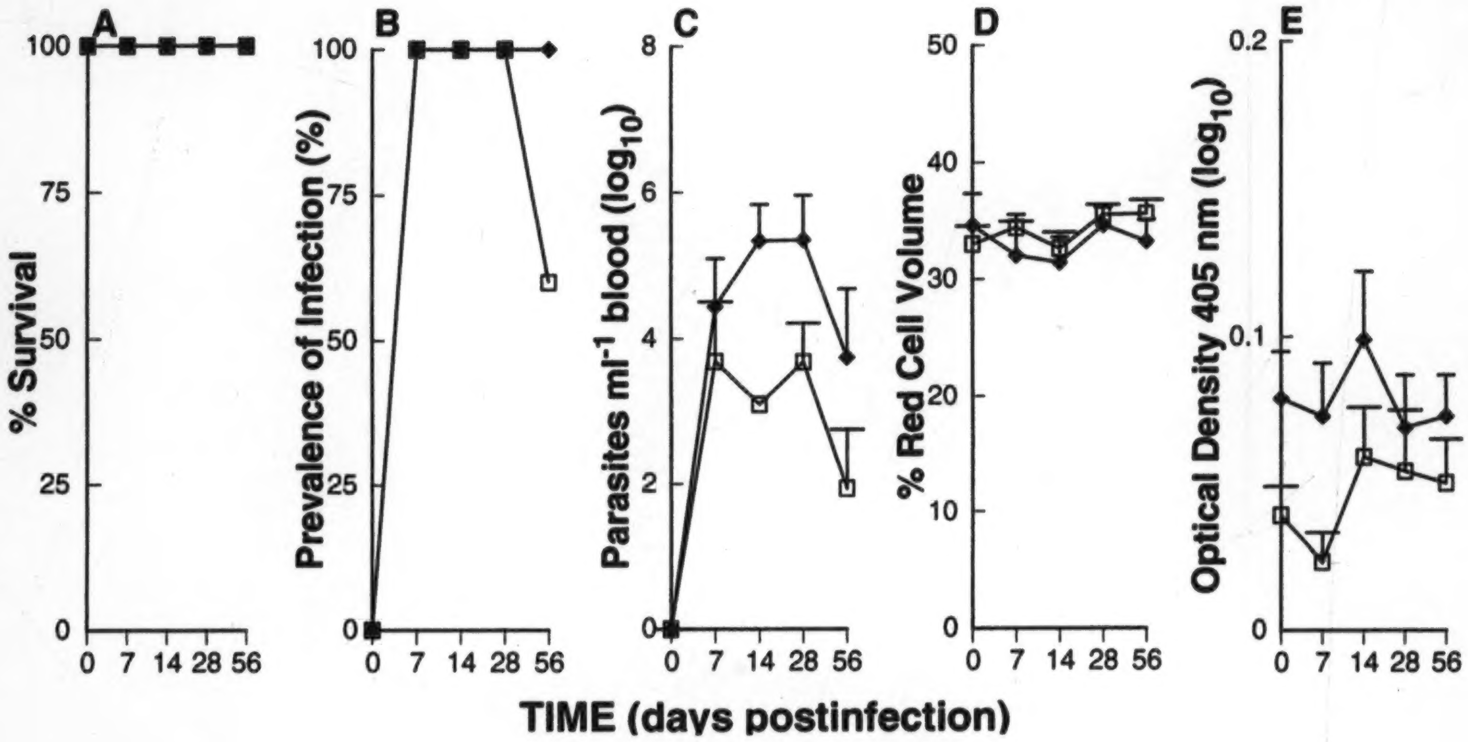

Fig. B-1. Effect of intravenous administration of ◆ trypanosome lysate or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

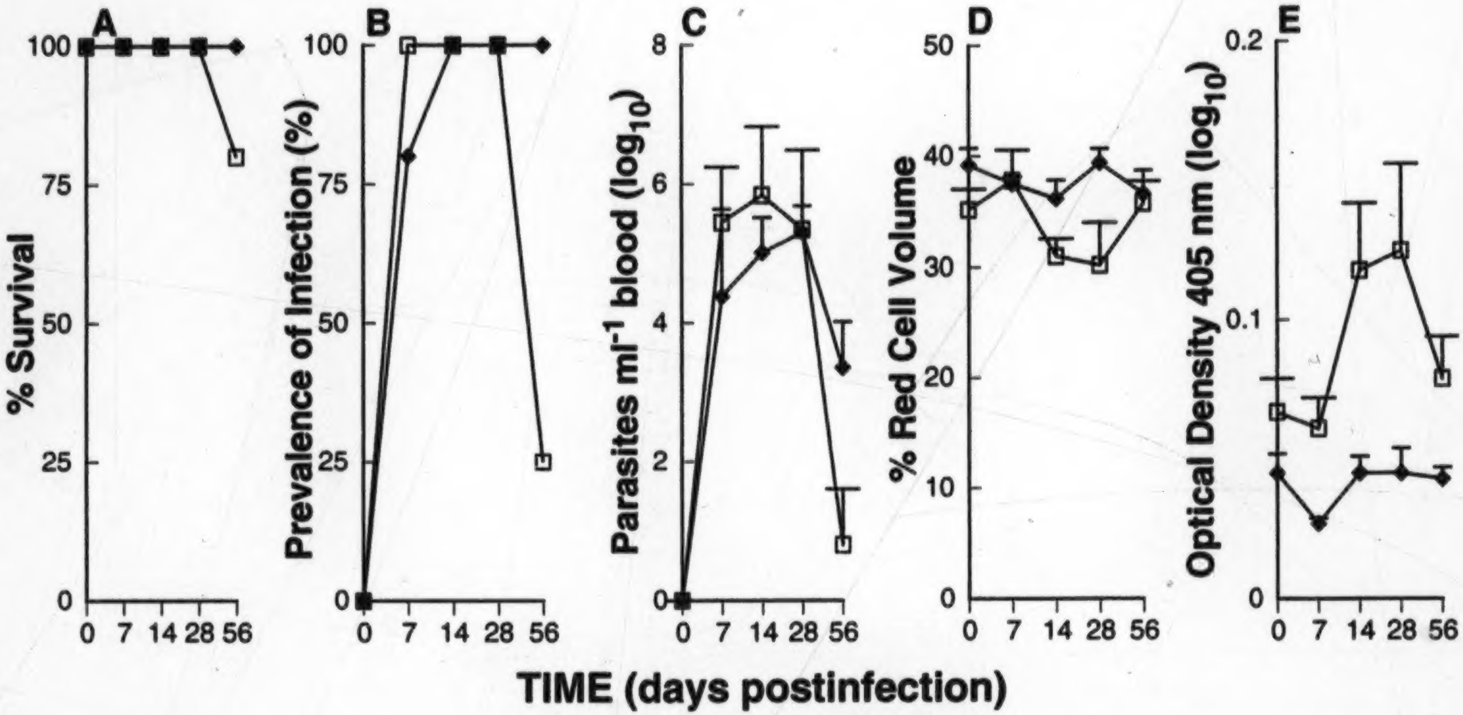

Fig. B-2. Effect of intramuscular administration of ◆ trypanosome lysate or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

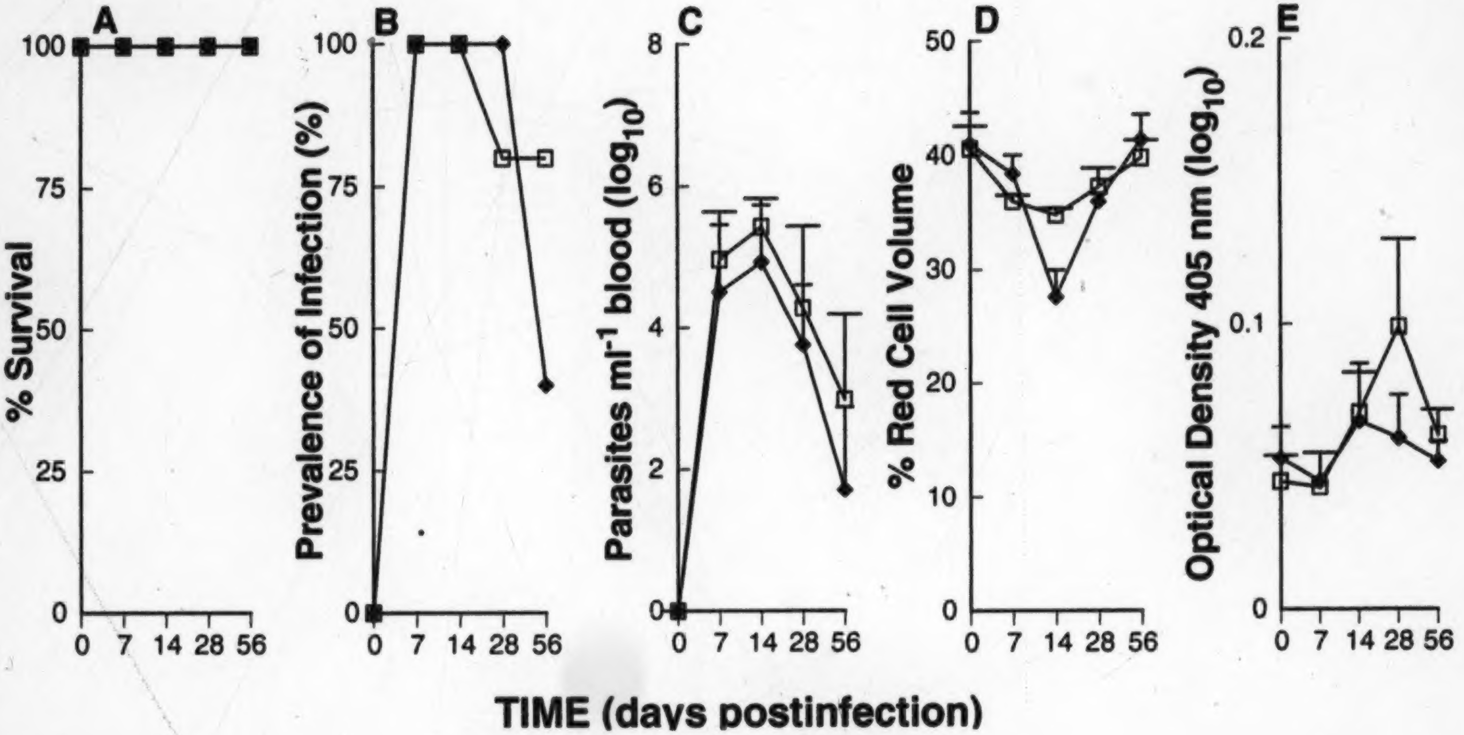

Fig. B-3. Effect of intraperitoneal administration of ◆ trypanosome lysate or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

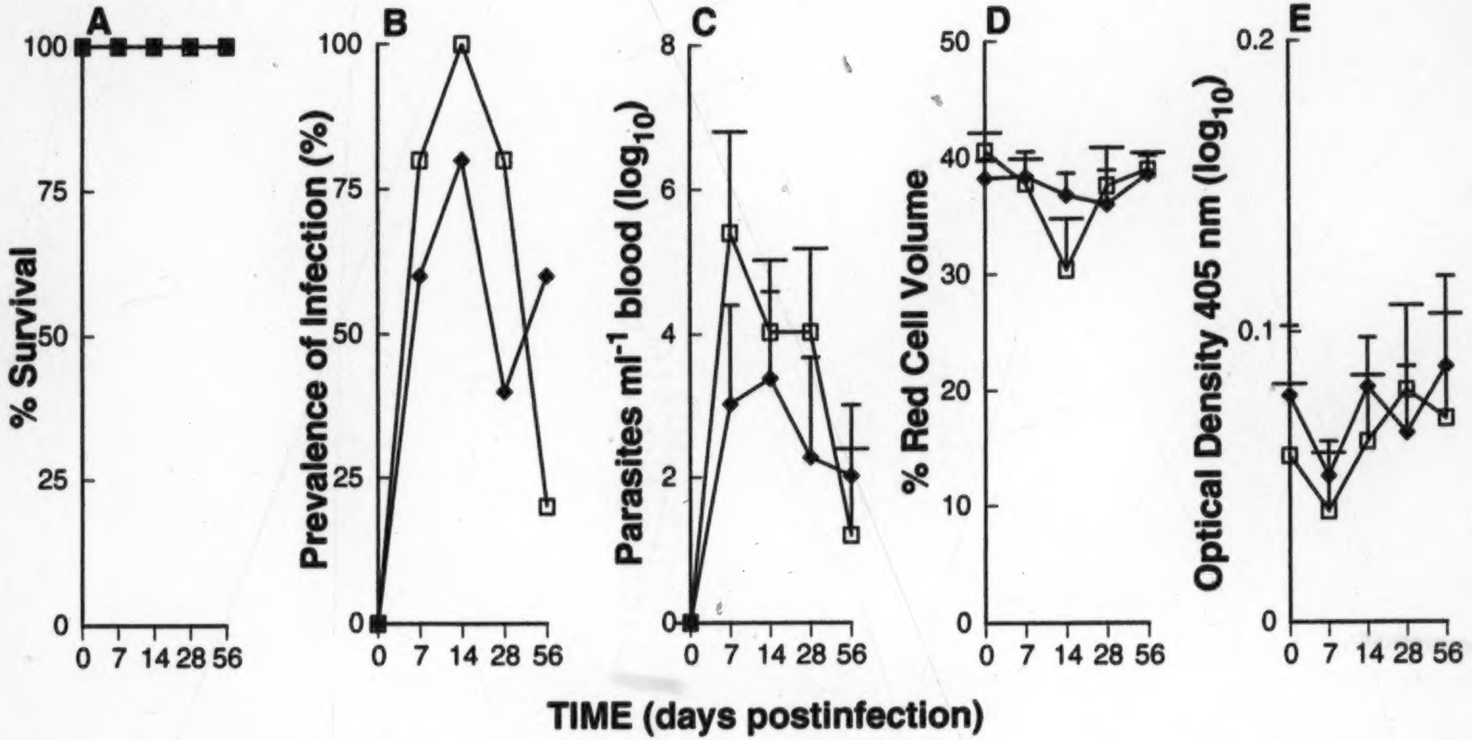

Fig. B-4. Effect of intraperitoneal administration of ◆ trypanosome lysate/Freund's incomplete adjuvant or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

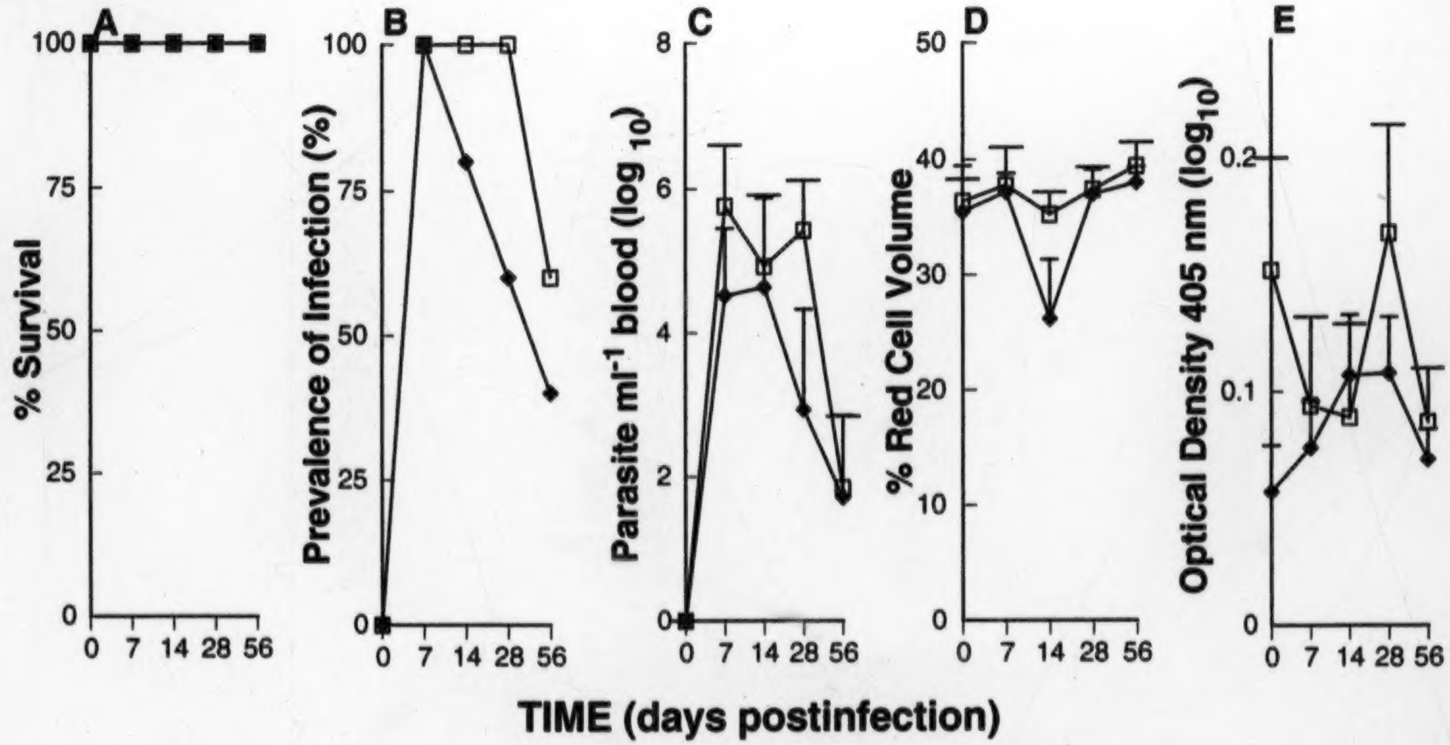

Fig. B-5. Effect of intraperitoneal administration of ◆ trypanosome lysate/Freund's complete adjuvant or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

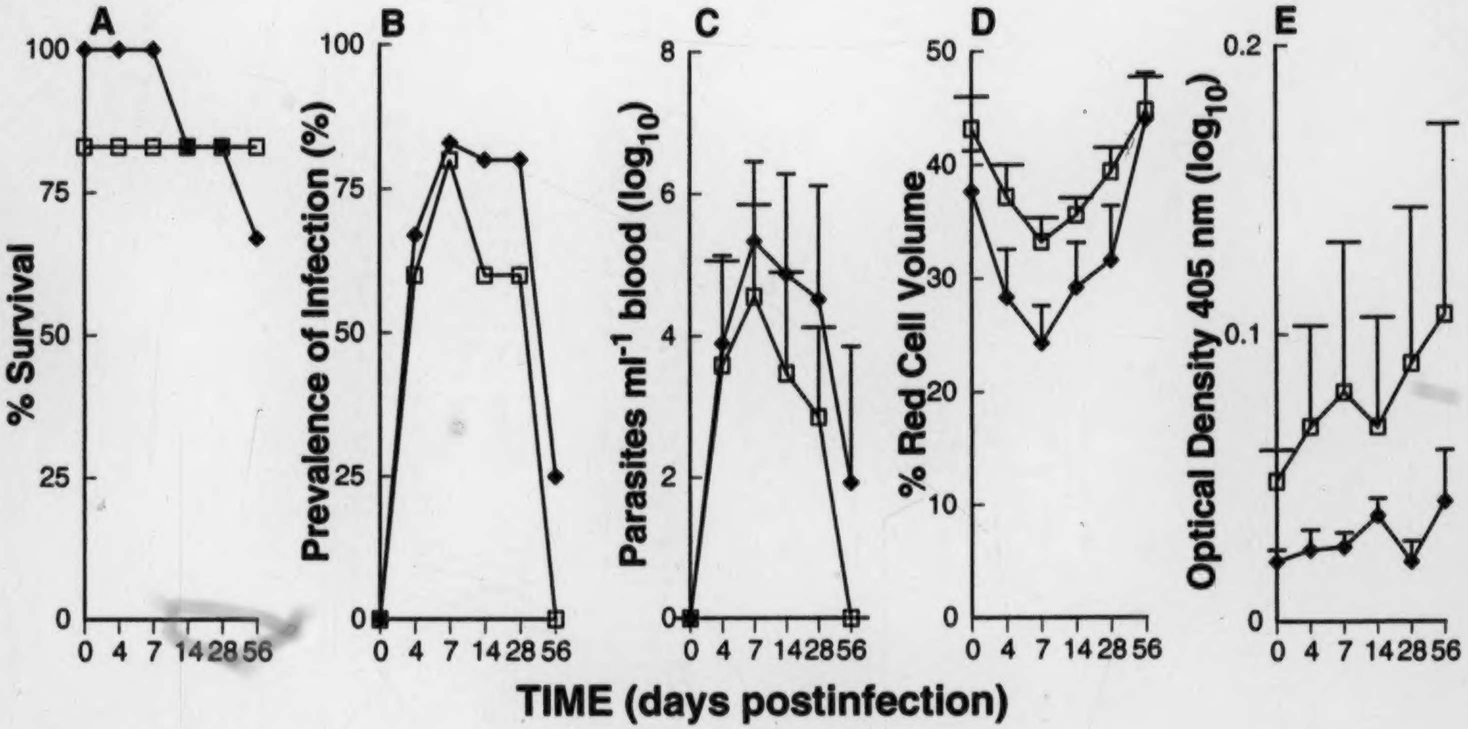

Fig. B-6. Effect of intravenous administration of ◆ water-soluble trypanosome molecules or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

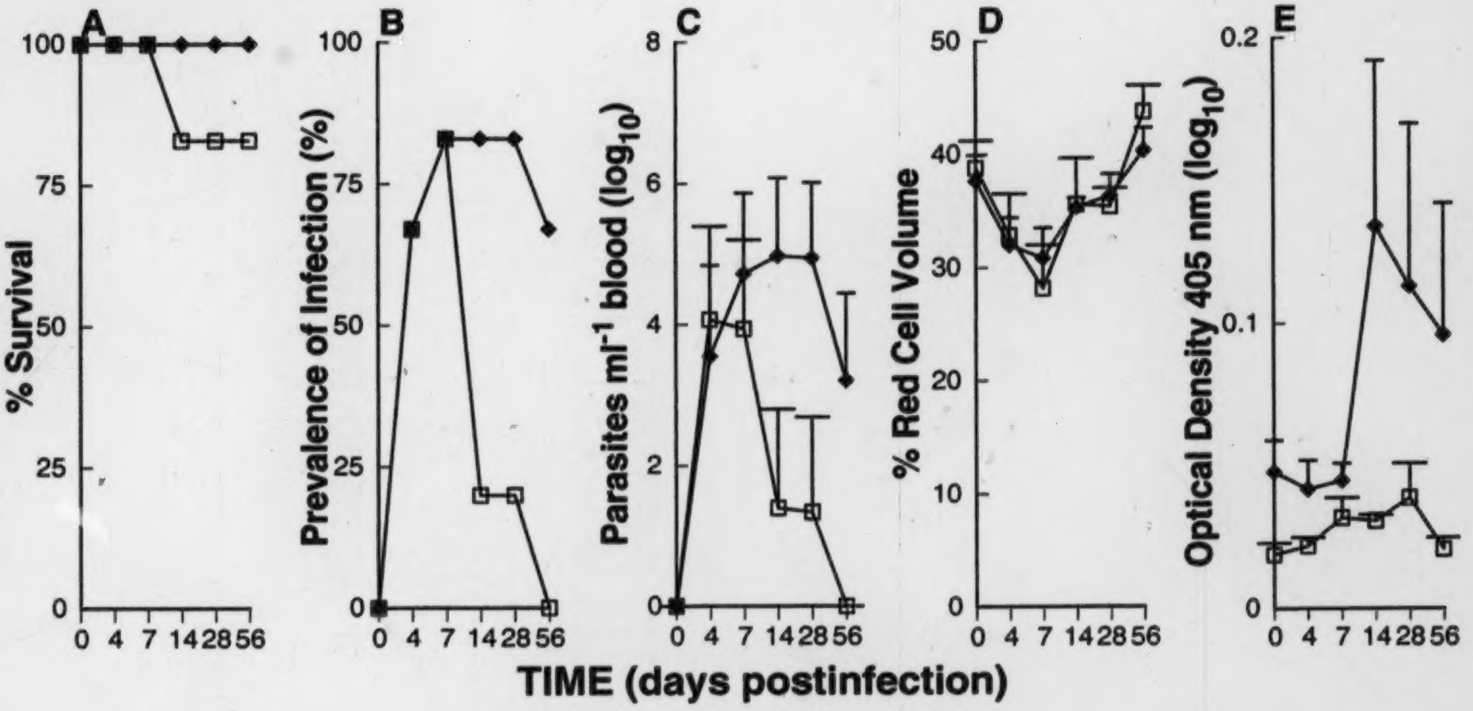

Fig. B-7. Effect of intraperitoneal administration of ◆ water-soluble trypanosome molecules or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

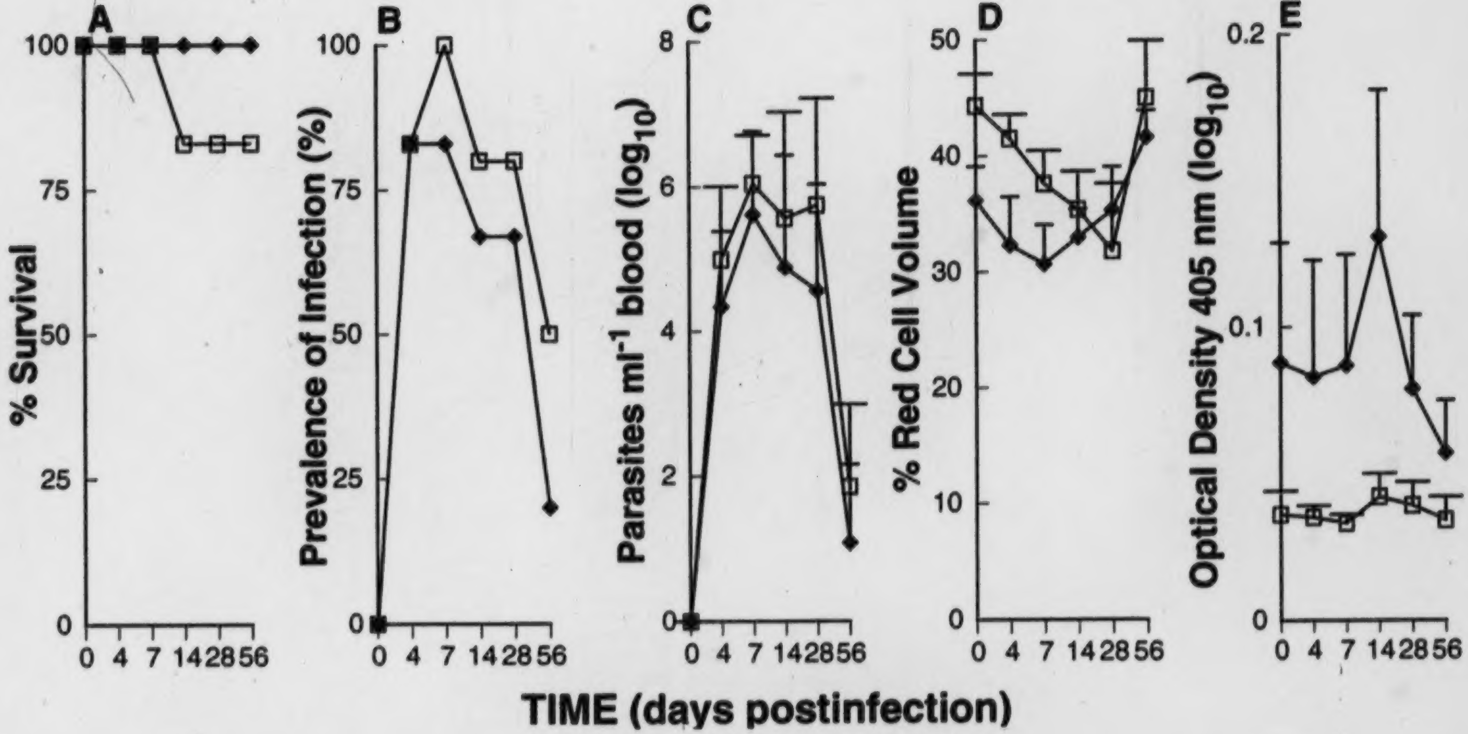

Fig. B-8. Effect of intraperitoneal administration of ◆ water-soluble trypanosome molecules/Freund's incomplete adjuvant or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

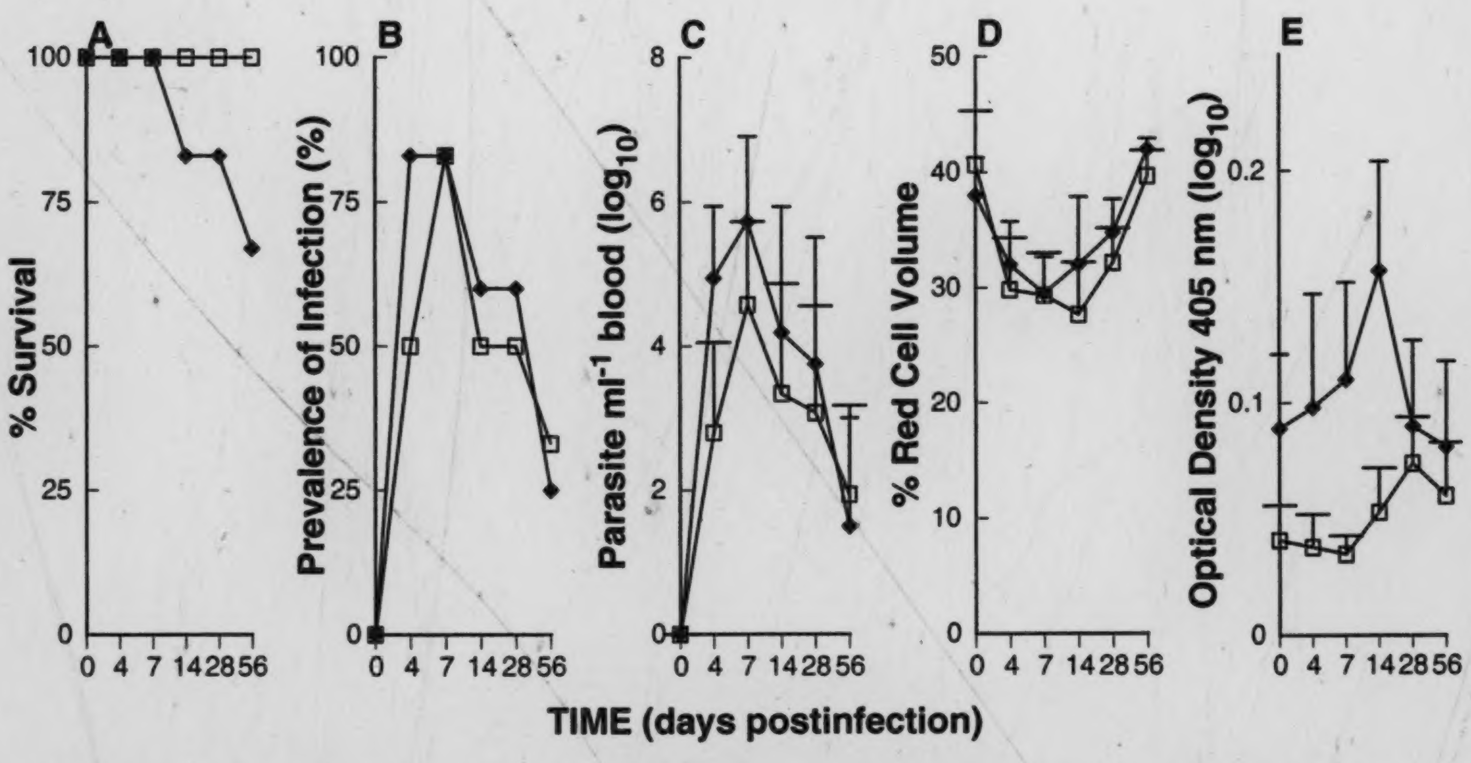

Fig. B-9. Effect of intraperitoneal administration of ◆ water-soluble trypanosome molecules/Freund's complete adjuvant or □ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells; E, relative antibody response. In figures C to E, data points represent the mean ± SEM.

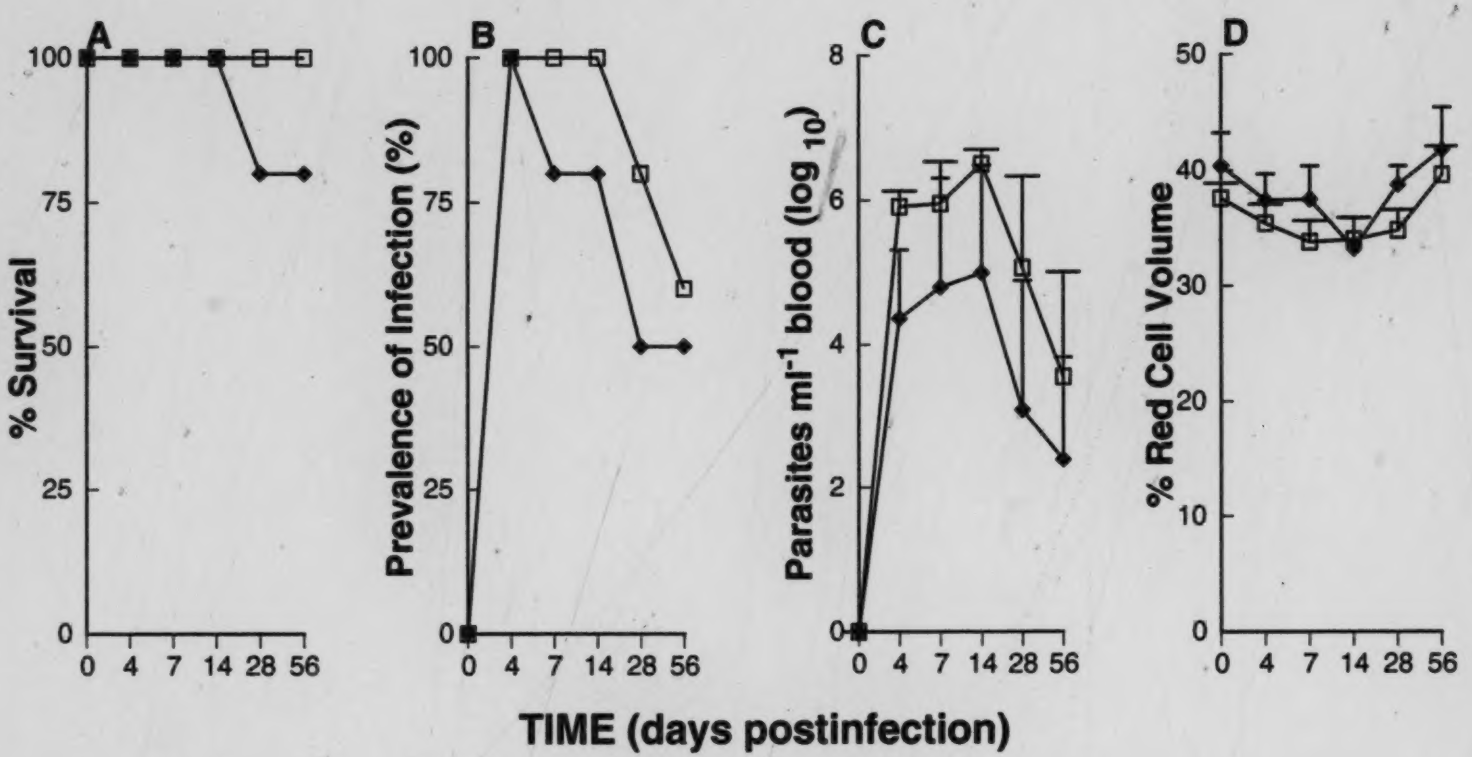

Fig. B-10. Effect of intravenous administration of ◆ detergent-soluble trypanosome molecules or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells. In figures C and D, data points represent the mean ± SEM.

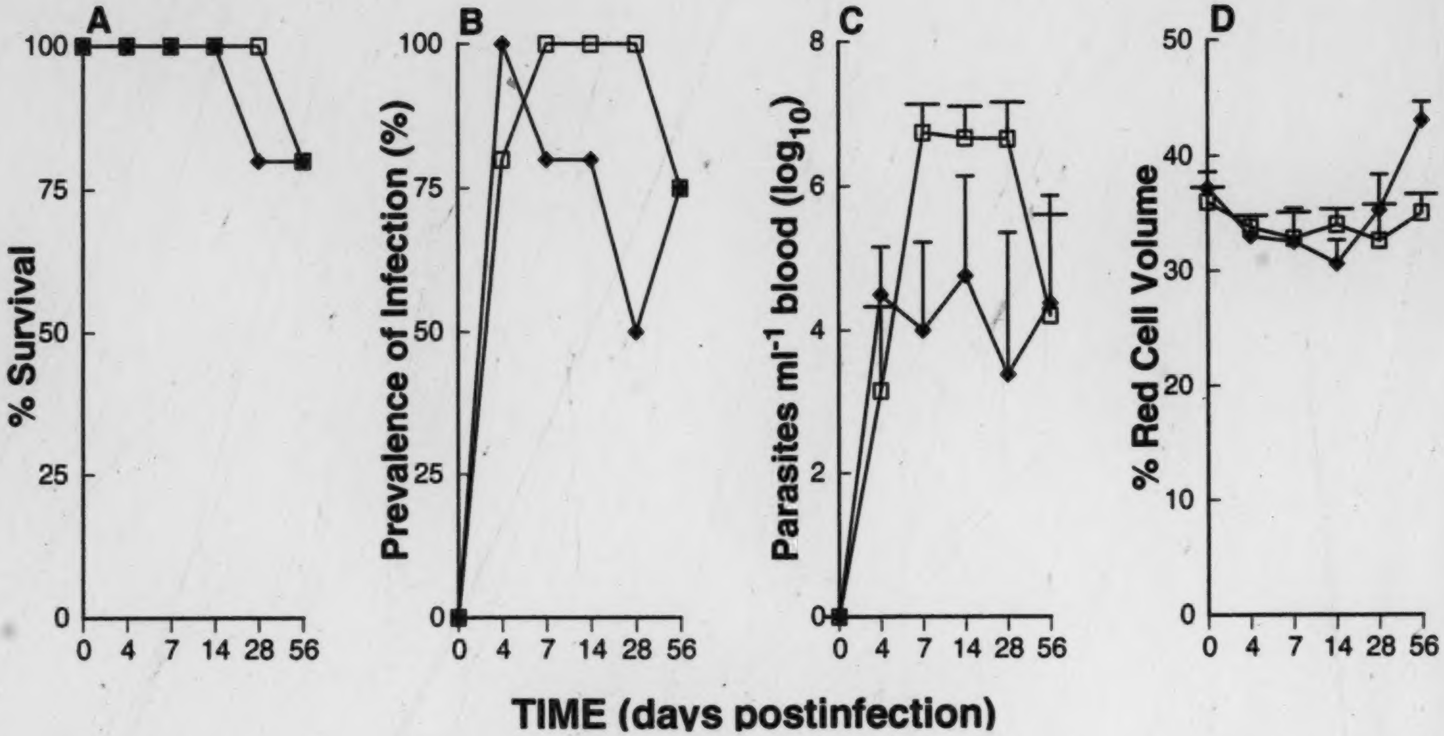

Fig. B-11. Effect of intramuscular administration of ◆ detergent-soluble trypanosome molecules or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells. In figures C and D, data points represent the mean ± SEM.

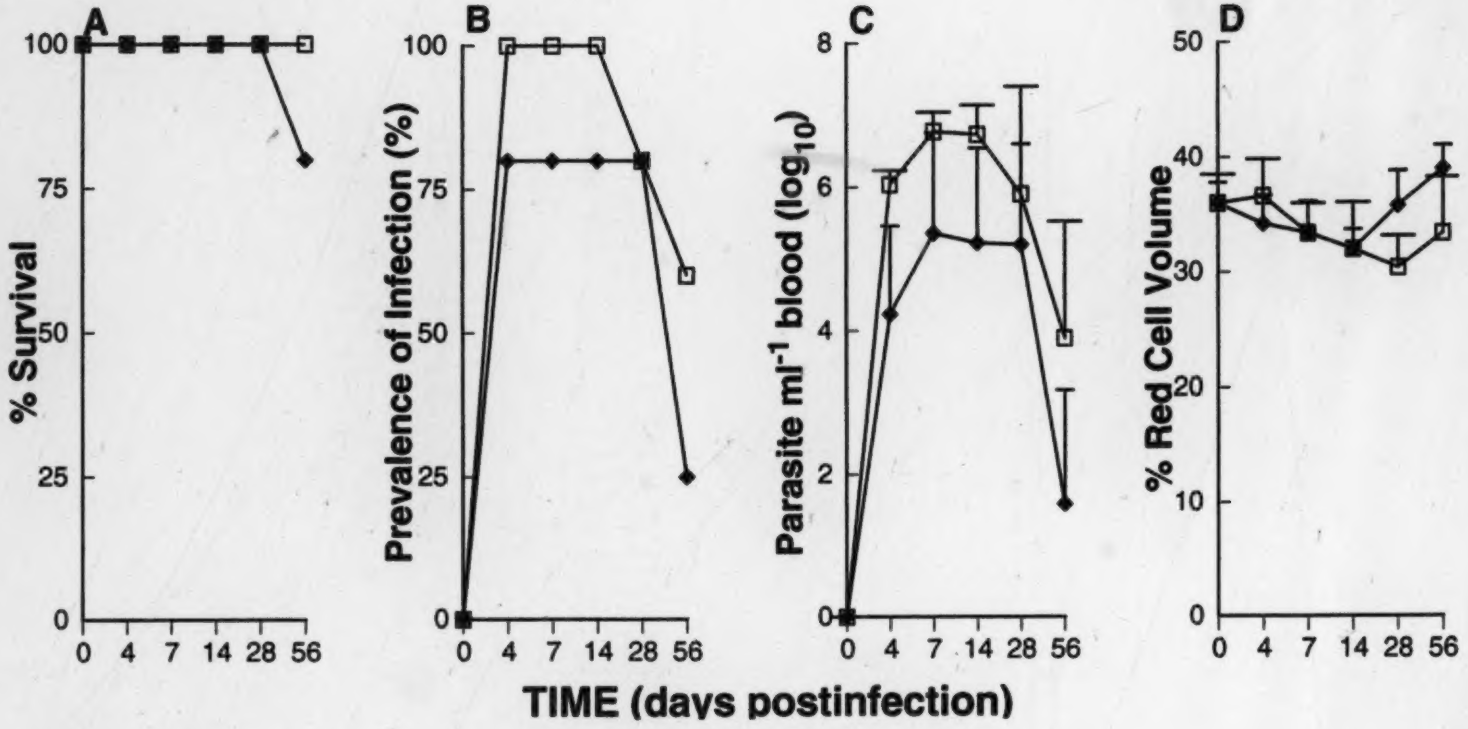

Fig. B-12. Effect of intraperitoneal administration of ◆ detergent-soluble trypanosome molecules or ☐ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells. In figures C and D, data points represent the mean ± SEM.

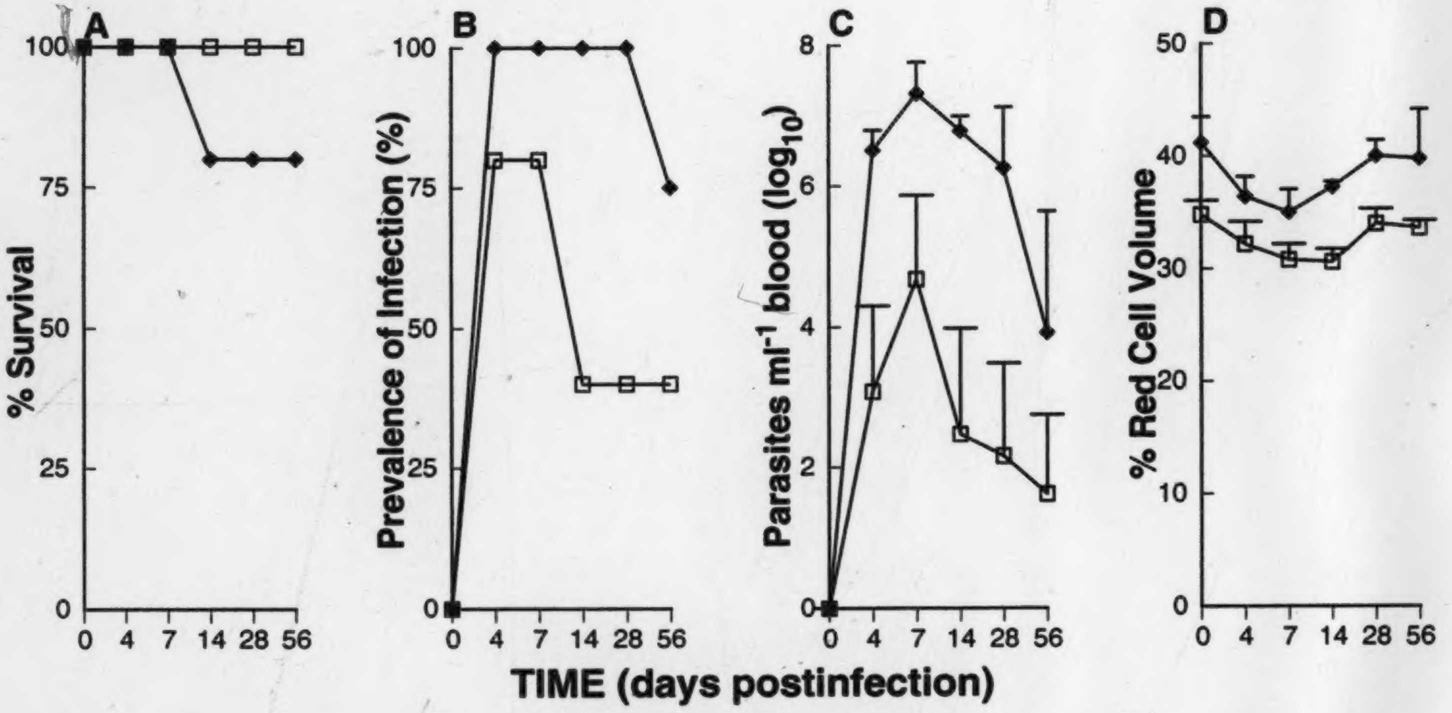

Fig. B-13. Effect of intraperitoneal administration of ◆ detergent-soluble trypanosome molecules/Freund's incomplete adjuvant or ☐ control solution, to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells. In figures C and D, data points represent the mean ± SEM.

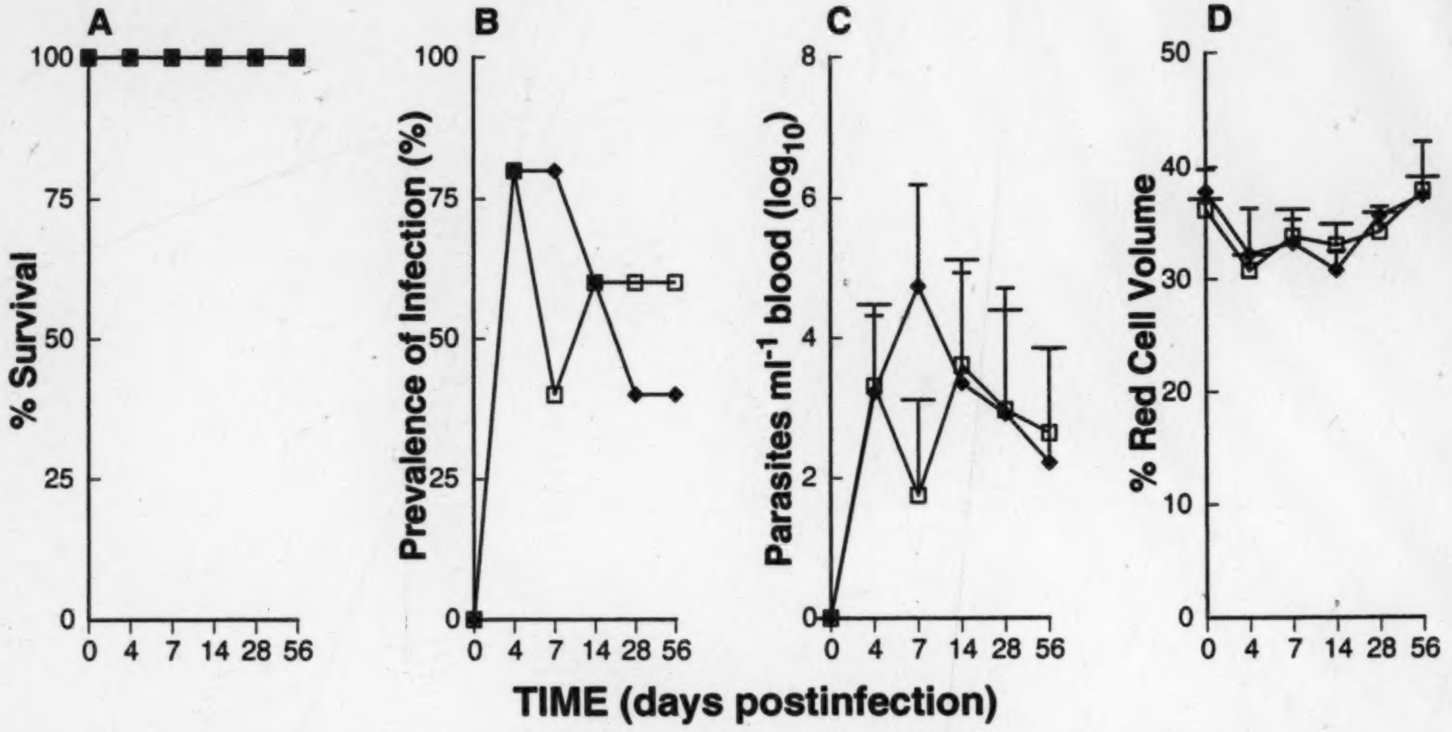

Fig. B-14. Effect of intraperitoneal administration of ◆ detergent-soluble trypanosome molecules/Freund's complete adjuvant or □ control solution to goldfish, prior to challenge with *Trypanosoma danilewskyi*. A, mortality; B, prevalence of infection; C, abundance of parasites; D, volume of packed red cells. In figures C and D, data points represent the mean ± SEM.

APPENDIX C

TABLE OF CONTENTS

Appendix C. *In vitro* study of interactions between macrophages and *Trypanosoma danilewskyi* 224
  Experimental design....224
    Mammalian macrophages....224
    Fish macrophages....224
  Results....225
    Mammalian macrophages....225
    Fish macrophages....225

IN VITRO STUDY OF INTERACTIONS BETWEEN MACROPHAGES AND *TRYPANOSOMA DANILEWSKYI*

EXPERIMENTAL DESIGN

Mammalian macrophages

Methods were adapted from Greenblatt *et al.* [117]. Approximately 6 X 10^4 viable P388D.1 cells were plated into each chamber of a Lab-Tek tissue culture chamber slide (Lab-Tek Products, Naperville, IL). After 18 hr of incubation at 37°C in a 5% CO_2 environment, nonadherent cells were removed by aspiration. The monolayers were washed with warm Dulbecco's PBS and DMEM containing 10% FBS and incubation was continued for 18 hr longer. Prior to addition of trypanosomes, monolayers were washed with warm Dulbecco's PBS and culture medium. One hundred microlitres of serum (diluted 1:10 in DMEM) was placed into each chamber followed by an equal volume of trypanosome suspension (~2.6 X 10^6 well^{-1}). Control chambers were handled in a similar manner, except that culture medium replaced the fish serum. Lab-Tek slides were incubated at 37°C in a 5% CO_2 environment for 1, 2, or 4 hr. After incubation, cells were washed extensively with warm Dulbecco's PBS. After removing the housing and gaskets, slides were rinsed and dried with forced air. Immediately thereafter, the slides were fixed for 60 s with methanol, air dried, and stained with Giemsa stain for 30 min. For each sample, 10 microscopic fields were examined (400 X magnification). Each parasite was recorded in one of two categories: 1) cells with only surface bound trypanosomes; and 2) cells with partially or entirely engulfed trypanosomes.

Fish macrophages

Goldfish kidney leukocytes were isolated from naïve and recovered goldfish (109 dpi) as described in Chapter II. According to the described procedures [203], the cultures were allowed to stabilize at 20°C. Thereafter, the cells were to be applied to the assay described in the previous paragraph.

RESULTS

Mammalian macrophages

Phagocytosis of *T. danilewskyi* was not significantly affected by the presence of serum (Fig. C-1). Accordingly, no significant differences were observed when samples containing control sera were compared to those containing immune serum. For all treatment groups, prolonged incubation increased the percentage of trypanosomes that were attached to or engulfed by macrophages. However, relative to the initial inoculum, only a minimal number (< 0.1%) of trypanosomes was associated with the macrophages. Certainly, this experiment was based on several assumptions (i.e., Fc-receptors of mammalian macrophages will bind fish IgM). For this reason, a subsequent experiment was attempted to determine if a greater extent of phagocytosis occurs with macrophages that were derived from recovered and naïve fish.

Fish macrophages

Unexpectedly, 10 to 12 days after isolation, low numbers of trypanosomes appeared spontaneously in macrophage cultures derived from recovered fish (data not shown). When viewed with the inverted microscope (200X magnification), the vast majority of trypanosomes were swimming freely in the medium. Notwithstanding, it was not uncommon to find a single trypanosome attached to a macrophage by the tip of the flagellum. Continued cultivation resulted in exacerbation of *T. danilewskyi* and eventually death of the macrophages. I wish to reemphasize that this occurred during the isolation and preparation of the macrophages. Consequently, the phagocytosis experiment was never conducted.

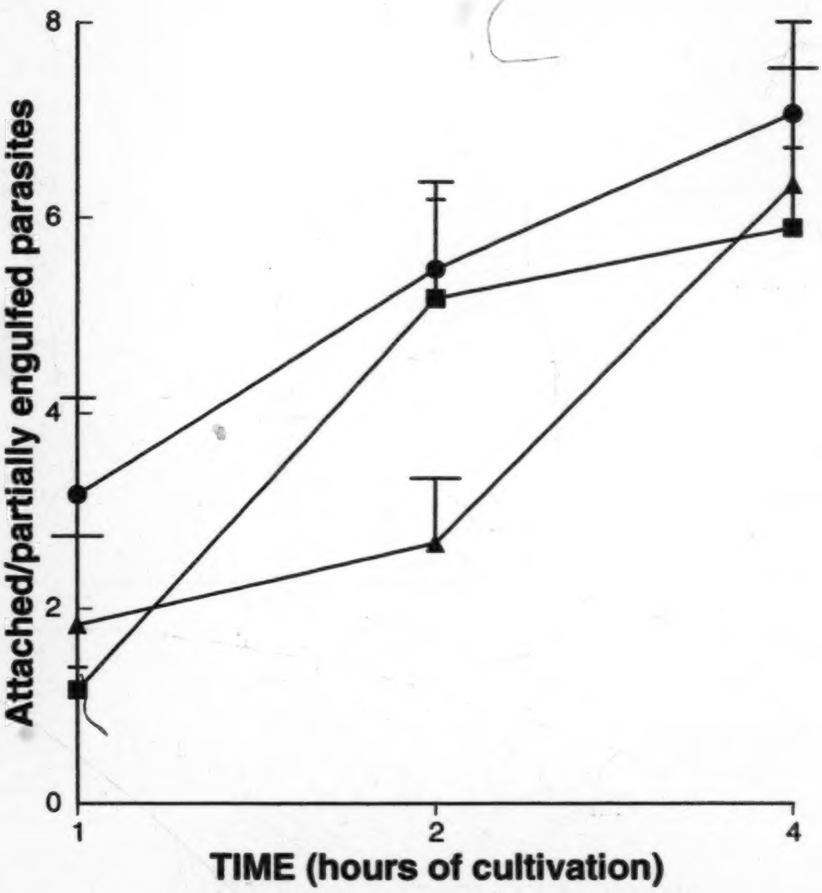

Fig. C-1. Interaction of *Trypanosoma danilewskyi* with mouse macrophages (P388D.1), after incubation in ■ immune or ● nonimmune fish serum. ▲, control chamber free of fish serum. Data points represent the mean number of attached or partially engulfed parasites ± SEM (n = 6).